The pharmacokinetics of D-penicillamine in normal and adjuvant arthritic rats

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THE PHARMACOKINETICS OF D-PENICILLAMINE IN NORMAL AND ADJUVANT ARTHRITIC RATS

Submitted by

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To mum and dad, for their unfailing love
and support throughout the years.

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ABBREVIATIONS

PSH	D-Penicillamine
PSSP	Penicillamine disulphide
PSSC	Penicillamine cysteine
AA	Adjuvant arthritis
RA	Rheumatoid arthritis
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
R_f	Relative to front
AUC	Area under the curve
tl/2	Biological half-life
DPM	disintegrations per minute

SUMMARY

The pharmacokinetics of D-penicillamine were investigated in normal rats and an experimental animal model of arthritis, namely the adjuvant arthritic rat. Total serum D-penicillamine (i.e. free, protein-bound and all disulphide metabolites) levels were assayed by means of an HPLC system coupled to a gold-mercury electrochemical detector. D-penicillamine was well absorbed from the gut, oral bioavailability being 68%. The elimination of the drug from serum was shown to be tri-phasic. An initial phase with a very short half-life of approximately 3 minutes, was followed by a an elimination phase with a half-life of 27 minutes. The final, slower phase had a half-life of 51 hours.

The major metabolite identified in the serum of the rat was the symmetrical penicillamine disulphide. Free D-penicillamine was initialy present at approximately 4% of the total but was undetectable after 3 hours. Penicillamine cysteine was not detected.

The kinetics of penicillamine cysteine (the major metabolite in human blood) and penicillamine disulphide (the major metabolite in rat blood) were examined after oral and intravenous administration of each diulphide. Oral bioavailabilty of penicillamine cysteine was 48.1% whilst penicillamine disulphide was poorly absorbed,

oral bioavailability being less than 3%. Whilst the kinetics of penicillamine cysteine closely resembled D-penicillamine kinetics, penicillamine disulphide was shown to be eliminated much faster than the parent drug. Almost 70% of the intravenous dose appeared in the urine within 4 hours of dosing as compared to 45% after an intravenous dose of either D-penicillamine or penicillamine cysteine.

The effects of D-penicillamine and penicillamine cysteine on the development of rat adjuvant arthritis were assessed. Neither compound was found to alter the course of the disease, nor was the metabolism of either compound affected by the disease. The relevance of these results with regard to the studies performed in man and the suitability of rat adjuvant arthritis as a suitable model for the screening of potential anti-rheumatic drugs are discussed.

CONTENTS

SECTION 1:

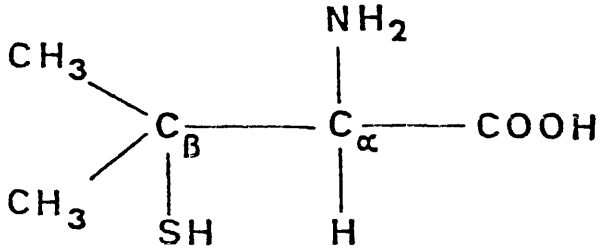
INTRODUCTION

The introduction to this thesis is divided into three sections. It begins with a summary of the background of D-penicillamine followed by a review of the work performed, to date, on the metabolism of the drug in man and animals. Finally, there is an overview of the experimental models of rheumatoid arthritis.

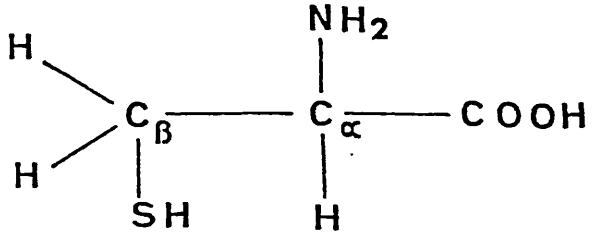
1.1. D-PENICILLAMINE

1.1.1 Structure and Chemistry of D-Penicillamine

D-Penicillamine was first isolated and identified in 1943 by Abraham and his coworkers during their attempts to elucidate the structure of penicillin (Abraham et al, 1943). This penicillin-hydrolysis product is a trifunctional amino acid, structurally similar to the naturally occurring amino acid, cysteine. It differs from cysteine in that two methyl groups replace the hydrogen atoms at the β- carbon, hence the generic name β,β-dimethylcysteine (Fig. 1.1). The asymetrical α carbon atom enables it to exist as either the D- or L- stereoisomer and, if synthesized from a racemic precursor, is present in both forms. The two enantiomers exhibit distinctly different metabolic and toxicological profiles (Kuchinskas et al, 1957) and it is the less toxic D form which is routinely used



D-Penicillamine



L-Cysteine

Fig. 1.1. Comparison of the Structures of D-Penicillamine and L-Cysteine

therapeutcally. D-Penicillamine is laevorotatory in solution, hence the proper designation D(-)Penicillamine. In aqueous solutions, penicillamine is slowly oxidised to penicillamine disulphide, this reaction being facilitated by the presence of air, traces of heavy metals light, raised temperatures and alkalinity (Doornbos and Feitsma, 1967).

As shown in Figure 1.1, D-Penicillamine posesses three funtional groups; a carbonyl, a sulphydryl and an amino group. These three different functional moieties enable D-Penicillamine to take part in diverse reactions characteristic of each group. These biochemical properties are summarised in Table 1. The capacity of D-Penicillamine to participate in such diverse reactions has lead to its use in the treatment of a variety of seemingly unrelated diseases

1.1.2. Therapeutic Applications of D-Penicillamine.

The first reported therapeutic use of D-Penicilamine was not until thirteen years after its discovery. Wilson´s disease (hepatolenticular degeneration) is a genetic disorder resulting in the excessive deposition of copper in tissues. In 1956 Walsche (Walsche 1956), realizing the metal chelating ability of D-penicillamine, speculated and subsequently proved the usefulness of the compound in the treatment of this

(a) Complex formation.

(i)	P-SH + heavy metals (Cu, Zn)	⟷	(PSH)-metal
(ii)	P-SH + pyridoxal	⟷	thiazolidine derivative

(b) Disulphide Formation.

(i)	P-SH + thiol-SH (P-SH, cysteine)	⟷	P-S-S-thiol
(ii)	P-SH + protein-SH	⟷	P-S-S-protein

(c) Thiol-Disulphide Exchange.

(i)	P-SH + disulphide (thiol-S-S-thiol)	⟷	P-S-S-thiol + thiol-SH
(ii)	P-SH + protein S-S-thiol	⟷	P-S-S-protein + thiol SH

(d) Methylation.

(i)	P-SH +CH_3 (methyl donor)	⟷	S-methyl penicillamine

(P-SH = D-penicillamine)

Table 1. Biochemical Reactions of D-Penicillamine.

disease. As a result of its ability to chelate copper, D-Penicillamine complexes with albumin bound copper releasing it from proteins thereby rendering it available for filtration at the glomerulus. Plasma copper levels are replaced from tissue stores and an equilibrium is reached between the protein-metal bonds in plasma and tissues (Kekki et al, 1966).

As with Wilson's Disease, the chelating ability of penicillamine has lead to its use in the treatment of certain heavy metal poisonings. The administration of D-penicillamine to hyperactive children with raised blood lead levels has proved to be therapeutically effective by promoting urinary lead excretion (Darrow and Schroeder, 1974). In their critical review of mercurial toxicity, MacGregor and Clarkson (1974) report that D-penicillamine is an effective oral antidote to mercuric chloride and that the acetyl derivative is also effective against mercury vapour intoxication. Although D-penicillamine can complex with iron, it does not deplete iron stores or reduce serum iron (Lyle, 1979). Serum zinc levels are thought to be unaffected but zinc depletion has been found to occur at a cellular level in patients with generalized scleroderma treated with penicillamine (Jepson and Eggert, 1984). Table 2 lists the therapeutic applications of D-penicillamine.

Usually Effective	**Usually Ineffective**
Wilson´s Disease Cystinuria Rheumatoid Arthritis Lead Poisoning Mercury Poisoning	Systemic sclerosis Cystinosis Ankylosing Spondylitis Psoriatic Arthritis Waldenstroms´s Macroglobulinaemia
Ineffective	**Disputed or Under Test**
Huntington´s chorea Duchenne muscular dystrophy Cadmium poisoning	Primary Biliary cirrhosis Schizophrenia Arsenic poisoning Antimony poisoning Darier´s disease Idiopathic pulmonary fibrosis Active chronic hepatitis

Table 2. Therapeutic Applications of D-Penicillamine (from Lyle, 1979)

In addition to his predictions about the value of D-penicillamine in the treatment of Wilson's Disease, Walsche (1956) also hypothesized that the capacity of penicillamine to take part in thiol-disulphide exchange reactions could be beneficial in the treatment of cystinuria, an hereditary disorder of amino acid transport resulting in the deposition of cystine crystals in the kidney. The mechanism of action of D-penicillamine in this disease is to react with cystine to form the more soluble compound, penicillamine-cysteine disulphide. The formation of this readily excretable disulphide reduces "cystine stone" formation in renal tubules. However , it was left to Crawhall and his coworkers to prove the rationale behind this theory correct (Crawhall et al, 1963).

The potential of penicillamine to cleave disulphide bonds was further demonstrated <u>in vitro</u> with macroglobulins (Ritzman et al, 1960). This finding led Jaffe to theorize that if penicillamine could cleave the macroglobulin rheumatoid factor (RF), the disease process might be interrupted. However, although a short course of injections of penicillamine into the knee joint lowered the titre of RF in the synovial fluid, it did not relieve the symptoms (Jaffe, 1962). Systemmic administration of the drug over several months

eventually brought about a reduction of serum RF, clinical improvement becoming evident first. Furthermore cessation of therapy was not followed by a return of pretreatment RF levels until many weeks later. It is now well established that any clinical improvement in rheumatoid patients in response to penicillamine is seen only after a latent period of several weeks. Also, whilst in some patients the response may be drammatic, others may show little or no improvement. In addition, there is an alarming incidence of adverse reactions amongst rheumatoid arthritis (RA) patients. The major side-effects of the drug will be dealt with later.

1.1.3 Possible Mechanisms of Action of D-Penicillamine in Rheumatoid Arthritis.

As previously discussed, D-penicillamine has an established role in the treatment of three unrelated disorders; Wilson's Disease, cystinuria and rheumatoid arthritis. In the first two, its mode of action is fairly well understood. However, in RA it remains speculative mainly because the pathogenesis and pathological dynamics of the disease are unclear.

Considering the number of biochemical reactions in which penicillamine might interfere there have been

many proposed mechanisms described for the therapeutic action of the drug. In active RA, serum copper and caeruloplasmin (the major copper carrying protein in plasma) are maintained at higher levels. These levels tend to fall towards normal when the disease activity is suppressed by penicillamine (Scudder et al, 1978). However, the ability of penicillamine to promote copper excretion is unlikely to be beneficial per se. Jayson et al (1976) have shown that serum copper and caeruloplasmin levels are simmilarly raised in ankylosing spondylitis, a disease in which penicilamine treatment is usually ineffective. Nevertheless displacement of copper from protein bound sites and subsequent complex formation and redistribution may be of importance.

Another proposed mode of action of D-penicillamine is the scavenging of oxygen derived radicals (ODR's) produced by phagocytes penetrating inflammatory sites. The hydroxyl ($OH^{\cdot}$) radical, generally considered to be the most reactive of these species, is formed from superoxide anion $O_2^{-\cdot}$ and H_2O_2 in the presence of transition metal ions, generally iron (Gutteridge et al, 1982). These radicals are deleterious for cell membranes and for the polysaccharides present in the synovial fluid (McCord, 1974). The chelate formed with penicillamine has been shown to have superoxide dismutating activity, thus the removal of the $O_2^{-\cdot}$ anion

necessary for $OH^{\cdot}$ formation (Greenwald and Moy, 1980) may account for some of the beneficial effects seen with D-penicillamine. Indeed, Sorenson (1976) proved that copper complexes of many anti-inflammatory drugs, including D-penicillamine, are more active than their parent compounds in several experimental inflammatory models. In addition, chelation of the iron that catalyses $OH^{\cdot}$ formation may also protect the joint from the destructive effects of these radicals. However, it should be remembered that chelation of iron by D-penicillamine is relatively poor in comparison with that seen with lead or copper ions (Doornbos and Faber, 1964).

Copper is also an essential requirement for the enzyme lysyl oxidase which is responsible for collagen cross-linking (Nimni, 1977). Since the concentrations of penicillamine required *in vitro* to inhibit this enzyme (and other enzymes with metals as cofactor) greatly exceed those reached *in vivo* (Siegel, 1977), interference with collagen cross-linking by this method seems unlikely. However, since penicillamine contains both a thiol and an amino group it can form lysyl and hydroxylysyl derived aldehydes (Nimni, 1977; Siegel, 1977). It has been proposed that the significance of this reaction is a stabilising effect on collagen making it less susceptible to degradation (Crawhall et al, 1979). Gerber (1978) proposed that D-penicillamine

may mimic the action of collagenase inhibitors due to disulphide exchange reactions thereby regulating collagenase activity in the rheumatoid joint. This same author has shown that, in vitro, the drug inhibits the sulphydryl dependent heat denaturation of IgG (Gerber, 1978) and so might prevent the formation of autoantigenic IgG aggregates in vivo. However, there is some dispute as to whether the levels of free D-penicillamine reached in plasma are sufficient to cause such an effect (Giacomello, 1981).

In addition to these biochemical effects, D-penicillamine has a profound effect on the immune response in the disease, suggesting an immunoregulatory mechanism of the drug. In vitro mitogen-induced lymphocyte proliferation can be enhanced at low doses of D-penicillaimne and inhibited at higher doses (Room et al, 1979). D-penicillamine has also been shown to synergise with copper ions in vitro to suppress lymphocyte function by inhibiting T-cell function (Lipsky and Ziff, 1980). This has been attributed to the generation of hydrogen peroxide by the penicillamine-copper complexes which suppress T-cell activity (Lipsky, 1984). However, no convincing T-cell activity in vivo by D-penicillamine has yet been shown. Evidence has been produced indicating a possible immuno-regulatory role for D-penicillamine in RA by affecting thiol-dependent reactions of monocytes

(McKeown et al, 1984).

This interference in the immune system may, in part, be responsible for a number of the adverse reactions observed with D-penicillamine. Coleman and coworkers (1986) have shown the existence of drug-specific antibodies against captopril, a thiol containing drug structurally related to D-penicillamine and whose side-effects are similar in many respects to those of D-penicillamine. As both drugs are extensively protein bound via disulphide linkages they may be acting as haptens to induce drug-specific antibody responses, thus leading to a state of hypersensitivity.

1.1.4. Adverse Effects of D-Penicillamine.

Commencement of penicillamine therapy in rheumatoid arthritis patients follows generally the same indications for the employment of chrysotherapy. Patients who are sufficiently active and who have failed to respond to treatment with non-steroidal anti-inflammatory drugs are candidates for treatment with penicillamine. In 1973 a multicentre trial not only demonstrated the efficacy of penicillamine as an anti-rheumatic agent, but also highlighted its capacity to induce the adverse reactions listed in Table 3. (Andrews et al, 1973). This is in sharp contrast to its

Common Side Effects

Gastrointestinal Upset	Early Rash
Diarrhoea	Thrombocytopenia
Buccal Ulceration	Leukopenia
Alterations in Taste	Proteinuria

Uncommon Side Effects

Aplastic Anaemia	Good Pasture's Syndrome
Systemmic Lupus Erythematosus	Febrile Reactions
	Myasthenic Syndrome
Nephrotic Syndrome	Pemphigus

Table 3. Adverse Reactions in D-Penicillamine Therapy.

use in Wilson's Disease and cystinuria where much higher doses are well tolerated. As a result of the alarming incidence of side effects in rheumatoid patients a dosage regimen of "go low, go slow" is generally employed. Patients are started on 125-250 mg daily and the dosage raised by degrees over several weeks until a maintenace dose (500 - 1000 mg daily) is reached at which the patient shows clear signs of improvement. The side effects, whilst being unpleasant, are mostly not serious and are reversible upon cessation of treatment. Amongst the commonest of problems encountered with D-penicillamine therapy are nausea, skin rashes and loss of taste all of which occur in approximately 25% of patients. "Early" and "late" rashes disappear on cessation of therapy and generally do not recur on recommencement of treatment. Taste usually returns whether therapy is discontinued or not. A more important complication is proteinuria and may lead to withdrawal of the drug. Its incidence and severity are variable and dose related - Crawhall (1981) reports proteinuria occurred in 30% of patients receiving in excess of 750 mg daily for more than six months. In some of these cases nephrotic syndrome can occur. It is essential to monitor renal function during penicillamine treatment. Haematological abnormalites can also occur, of which thrombocytopenia is the most common. When this occurs, treatment is usually discontinued to facilitate recovery but can generally

be cautiously restarted. More serious effects (but fortunately less common) include neutropenia, blood dyscrasias and drug-induced systemmic lupus erythematosus (SLE).

The precise reasons for such inter-individual variations in both efficacy and toxicity to D-penicillamine are as yet unknown. However, it may be in part be related to plasma levels of the drug and/or its metabolites. In order to test the validity of this hypothesis, a precise and accurate method of analysis for D-penicillamine and its metabolites is a pre-requisite.

1.1.5 Assays for D-Penicillamine in Biological Fluids.

Pharmacokinetic studies have so far been hampered by the lack of reliable, sensitive and selective methods for the determination of D-penicillamine and its metabolites. The inherent reactivity of penicillamine has proved to be a considerable problem. Only in recent years have procedures been adopted which have eliminated problems in penicillamine analysis. These have been mainly the use of EDTA to chelate any metal ions present, thereby preventing penicillamine chelation, and manipulation of the pH to keep the drug in the desired form (Bergstrom et al, 1980). The

analytical methods employed to date can be separated into two groups: non-chromatographic and chromatographic. The first category encompasses spectrophotometry, which is limited by its lack of sensitivity and selectivity (Pal, 1959), and radioimmunoassay (RIA). The use of RIA is restricted by the relative unavailability of radiolabelled D-penicillamine and its metabolites required to produce specific antisera (Assem and Vickers, 1974).

Determination of penicillamine levels by chromatographic techniques such as amino acid analysis with ninhydrin detection (Friedman et al, 1977), and gas-liquid chromatography (Jellum et al, 1969) may provide quantitative information about the metabolism of the parent drug and its metabolites. However, resolution of these compounds from other amino acids requires time consuming analysis programmes for physiological fluids. In general this renders these methods unsuitable for large sample investigations such as those required for pharmacokinetic studies. Also, they assume that the metabolites are both known and are available as standards.

In recent years the convenience and versatility of high performance liquid chromatography (HPLC) has lead to its acceptance as one of the most useful techniques available for the analysis of drugs in biological

fluids. Due to the lack of a chromophore in the penicillamine molecule, low concentrations cannot be detected in the ultraviolet region. However, in recent years several HPLC methods using electrochemical detection have been described (Saetre and Rabenstein, 1978; Bergstrom et al, 1981 and Kreuzig and Frank, 1981). Although these methods are reliable and rapid, none of them allows the direct determination of individual metabolites. All the methods rely on the reduction of all penicillamine moieties (ie. all metabolites and protein bound penicillamine) back to the free thiol. To date, the direct measurement of individual penicillamine metabolites by a quick, reliable method remains to be perfected. Such an assay would permit full metabolic profiles for rheumatoid arthritic patients to be determined and correlation studies may shed light on the on the mechanisms by which penicillamine exerts its clinical benefit and produces side effects. In addition there is no satisfactory analytical procedure to measure the only truly "metabolised" form of penicillamine - the S-methyl derivative. This metabolite has been detected in plasma (Muijsers et al, 1979) and urine (Perret et al, 1975). In this project "total" d-penicillamine is measured by a modification of the method described by Abounassif and Jefferies (1983) employing a gold-mercury electrochemical detector specific for thiols. This method requires that samples must be

initially treated with the reducing agent Di-thiothreitol (Cleland's Reagent; Cleland, 1964). The oxidised disulphide forms of D-penicillamine are thus reduced to the SH-form, suitable for measurement by the HPLC system.

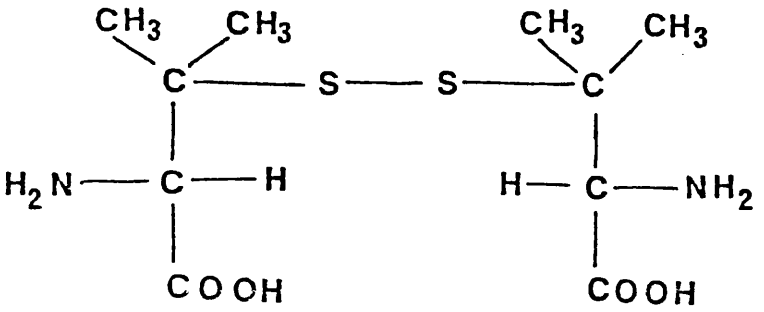
1.2. THE METABOLISM OF D-PENICILLAMINE.

As discussed previously, the recent development of more sensitive, reliable assays for D-penicillamine in biological fluids and tissue has allowed greater in depth studies on the metabolism of D-penicillamine. Pharmacokinetic studies of the drug and its metabolites may be important when trying to correlate the incidence of side effects or variation of efficacy to circulating levels of the drug or metabolites.

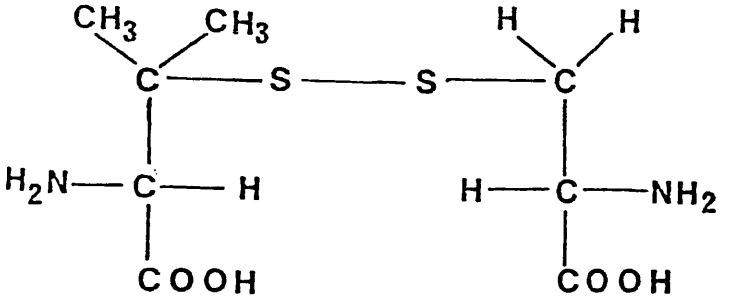
The hitherto identified metabolites of D-penicillamine are shown in Figure 1.2. The metabolism of the drug is predominantly governed by its ability to interfere with thiol-disulphide exchange reactions. Since the sulphydryl group is sterically hindered, the rate of equilibration with other disulphides is low in comparison to for instance cysteine. A less rapid incorporation of D-penicillamine into disulphides *in vivo* presumably makes it more effective for therapeutic purposes (Friedman, 1977).

1.2.1. Absorption.

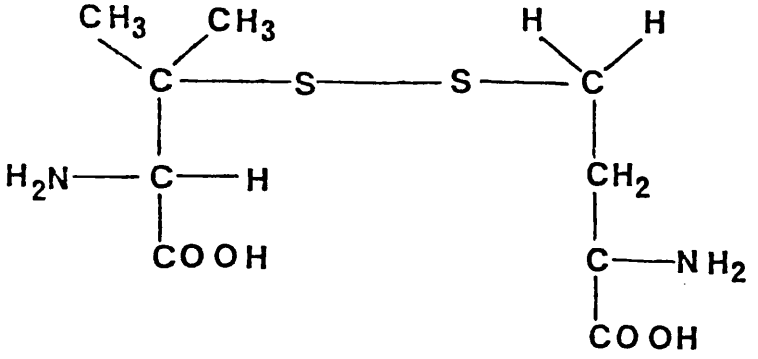
In man, oral doses of D-penicillamine are rapidly absorbed from the gut, peak serum concentrations occurring 2 - 3 hours after oral administration. The



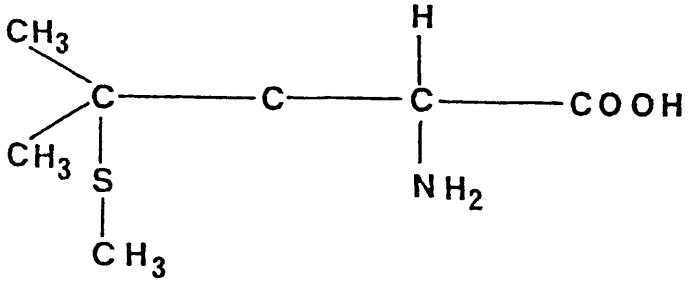
Penicillamine Disulphide



Penicillamine-Cysteine Disulphide



Homocysteine-Penicillamine Disulphide



S-Methyl D-Penicillamine

Fig. 1.2. Chemical Structures of Known Penicillamine Metabolites.

serum peak concentration is directly dose related (Muijsers et al, 1984; Bergstrom et al, 1981; Butler et al, 1982).

Wass and Evered (1970), on the basis of *in vitro* studies with inverted sacs of rat intestine, suggested that D-penicillamine required a specific carrier for transport across the gut wall. The importance of an intermediate stage in the absorption of thiols, including D-penicillamine, has been demonstrated by Perret (1981). Using intestinal perfusion techniques, the luminal disappearance of D-penicillamine was shown to be far greater than that of its disulphide and comparable to that of other thiols such as cysteine and glycine. The high disappearance rate is thought to be due to the result of rapid binding, via disulphide bonds, to the proteins of the gut wall. Following the formation of the disulphide bridge, the D-penicillamine could then transfer into the portal blood stream. As thiols compounds can physically damage the gut mucosa, and that the gut experiences the highest concentration of D-penicillamine, this may explain the large incidence of gastrointestinal upsets.

Absorption of D-penicillamine is probably influenced by its binding not only to gut proteins but also to proteins present in food. Oxidation to the disulphide would also reduce uptake. Planas-Bohne (1972) reported

that fasting rats absorbed 25% more D-penicillamine than did animals given free access to food. Perret (1981) also found that rheumatoid patients absorbed, on average, 50% more of the drug in the fasting situation than when it was taken with food. Muijsers et al (1984) also claim that taking D-penicillamine 2 hours after dinner resulted in even lower absorption than when taken with food. They also speculate that concommitant iron therapy reduces the absorption of penicillamine, although earlier work by the same research group failed to show any effect of simultaneous supplementation of iron sulphate (525 mg/day) on serum levels or renal excretion of the drug (van de Stadt et al, 1979). Schuna and coworkers (1983) reported that although the systemic availability of D-penicillamine is reduced, the rate of absorption of the drug is not.

1.2.2. Distribution.

In experiments using ^{14}C-labelled D-penicillamine in rats, a biphasic loss of plasma label occurred (Patzchke and Wegner, 1977; Planas-Bohne, 1981) and similar results have been obtained from studies which measure total D-penicillamine in human volunteers, showing a rapid elimination phase (half-life of about 1-5 hours) and a slower phase. Patzschke et al (1977b)

reported the half-life of the second slower phase to be 8 days, whilst Muijsers et al (1984) quote their findings of between 4-6 days.

The bi-phasic distribution profile shown by D-penicillamine would suggest the existence of two pools. The fast pool is thought to account for approximately 85% of the excretable D-penicillamine, whereas the slow pool is thought to be the reserve from which the serum pool is constantly refilled (van der Korst et al, 1981). Muijsers and coworkers (1984) report that the basal serum D-penicillamine levels gradually increase during prolonged therapy and slowly decrease upon termination of treatment. They tentatively suggest there may be a direct link between the slow onset of therapeutic effect of D-penicillamine in rheumatoid arthritis and the slow accumulation of a deep pool in the body. A possible location of this pool may be connective tissue. Animal studies of the distribution of labelled D-penicillamine show that the label is rapidly cleared from the liver and kidneys, but is cleared only slowly from collagen and elastin rich tissues, such as skin and bone (Patzschke and Wegner, 1977a).

In his review of the metabolism of D-penicillamine, Perrett (1981) concluded that, in humans, 80% of the serum D-penicillamine is protein bound, about 12% in

the disulphide forms and 6% is free penicillamine. The final 2% is probably in the form of metal complexes or as S-methylated D-penicillamine. However, these are the results mainly from studies on cystinuric patients and RA patients stabilised on D-penicillamine. Studies measuring free D-penicillamine levels after a single oral dose (Wiesner et al, 1981; Bergstrom et al, 1981) suggest that free D-penicillamine constitutes around 25% of the total. The inherent reactivity of D-penicillamine may explain this apparent discrepancy, ie. as the drug enters the body approxomately 25% remains in the free form. However, as more drug enters the plasma and the longer it remains there, the more gets oxidised, causing a drop in the level of the free drug to a steady basal level, eg 6%.

Another possible explanation is the method of measurement of free drug. The acid precipitation method (Saetre and Rabenstein, 1978) was used to obtain a non-protein fraction in which to measure free drug. This assumes that D-penicillamine is protein-bound via covalent disulphide bridges, unaffected by acid treatment. However if a proportion is loosely ionically bound, this would be released and maintained in the free form by the acid pH, and subsequently measured as free drug.

The lack of an analytical system to measure individual

metabolites has lead to some confusion as to which of the metabolites predominates. Most research groups so far have assumed that there is a mixture of penicillamine cysteine and penicillamine disulphide, the penicillamine cysteine predominating because of the low equilibrium constant for the formation of penicillamine disulphide (Jellum and Skrede, 1976). However, in a study by Kyogoku et al (1982) whereby they measured individual metabolites by the ninhydrin colorimetric method using an amino acid analyser, they reported that the main human metabolite was penicillamine-cysteine and that penicillamine disulphide was not detectable. In addition to this, they showed that species differences in metabolites occur. For instance, although the rat and dog show the same biphasic kinetic profile as man, both animals also have detectable levels of penicillamine disulphide.

The formation of disulphides is considered to be a dynamic process. In a short report by Miners (1984) he shows that measurable concentrations of penicillamine were present in plasma after intravenous and oral administration of both penicillamine cysteine and penicillamine disulphide to rats. This demonstrates the in vivo reduction of disulphide metabolites back to the parent drug. The enzyme(s) involved in this reduction are not known, but Miners (1984) suggests the involvement of glutathione reductase, thiol-disulphide transhydrogenases and nonspecific disulphide

reductases. Moreover, although penicillamine is highly protein bound (mainly to serum albumin) it is not thought to be tightly bound and readily dissociates (Bergstrom et al, 1981b). In addition, the binding of D-penicillamine to serum proteins is not apparently dose related since higher doses of the drug lead to a decrease in percentage binding. This suggests the presence of a limited number of protein sites to which D-penicillamine can bind (van de Stadt et al, 1979). It is possible, therefore, that a dynamic equilibrium exists between free penicillamine, bound penicillamine and disulphide metabolites. This phenomena may be important if, as many believe, the active form of the drug is free penicillamine.

1.2.3. Excretion.

Studies by Perrett and his coworkers (1975) reported that free D-penicillamine was not detected in the urine or faeces of patients with rheumatoid arthritis or cystinuria. Penicillamine disulphide was present in both groups, whilst penicillamine cysteine was only present in the urine. In addition, the urine contained the only metabolically transformed metabolite, S-methyl-D-penicillamine. The total recovery of the drug in urine plus faeces, however, was less than 50% of the administered dose. In a later study in which Perrett himself took D-(2-^{14}C)-penicillamine, 35%

appeared in the faeces, which suggests bacterial degradation of D-penicillamine before full intestinal absorption could take place (Perrett, 1977). Another theory for low recovery of D-penicillamine in urine and faeces was proposed by van de Stadt et al (1979), whereby the S-methyl-D-penicillamine is oxidatively deaminated by D-aminoacid oxidase, an enzyme active in liver and kidney for the detoxification of D-aminoacids. Although the possibility exists that D-penicillamine may be enzymatically decarboxylated, a study by Kucharczyk et al (1984) in which they measured $^{14}CO_2$ expiration from rats after an oral dose of ^{14}C-D-penicillamine, reported that only 1.5% of the administered dose was expired. This indicates that the in vivo decarboxylation of the drug in rats may not be a significant metabolic pathway.

A further metabolite has been tentatively identified in the urine of patients with RA (Perret, 1981). Since homocysteine-cysteine disulphide is present in plasma and excreted in excess in cystinuria and Wilson's disease it is not an unreasonable supposition that homocysteine-penicillamine disulphide may be a penicillamine metabolite.

Patzschke et al (1977b), again using radiolabel studies in healthy volunteers, report 40% recovery of the label in the urine after oral dosing, and that the renal

excretion rate was maximal shortly after intake of the drug and drastically declined within the first 12 hours. Also the percentage of the drug that is excreted in the urine did not increase with dose or continued treatment suggesting that the "deep" pool is not easily saturated. Most studies agree that a total of 80% of the final dose detected in the urine is excreted in the first 12 hours.

The inability of most studies to detect free D-penicillamine in urine is probably due to the influence of urinary pH and incubation time (Carruthers et al, 1983). Hence any free thiol appearing in the bladder may be spontaneously oxidised to disulphides before excretion.

As yet no research group has been able to find a correlation between serum D-penicillamine levels and efficacy and/or toxicity. In a clinical trial undertaken by Munthe et al in 1979, it was found that the addition of cysteine to D-penicillamine treatment in non-responder RA patients raised the level of erythrocyte glutathione and, in some cases, turned non-resonders into responders. In relation to this, a preliminary report by van de Korst et al (1981) showed that a correlation existed between serum cysteine depletion and D-penicillamine induced side-effects.

However, this has been somewhat invalidated by their more recent study on a larger group of patients (Muijsers et al, 1984).

A recent study (Emery et al, 1984) has tested the ability of RA patients on comparable doses of D-penicillamine to oxidise S-carboxymethyl L-cysteine (carbocysteine). Patients were classified as either poor "sulphoxidisers" or extensive "sulphoxidisers". There was a significant association between impaired sulphoxidation and manifestation of toxic side-effects to D-penicillamine. Although these workers point out the structural similarity between D-penicillamine and carbocysteine, the similarity between the latter and S-methyl D-penicillamine is more marked. If this is the metabolite responsible for toxicity, patients unable to oxidise it may produce side-effects. Should this be true, then increased levels of S-methyl D-penicillamine in the plasma might usefully predict the development of toxicity as would classification of the patient's sulphoxidation status.

1.3. EXPERIMENTAL MODELS OF RHEUMATOID ARTHRITIS.

The development of a truly representative animal model of rheumatoid arthritis has been been seriously impaired by the inadequate knowledge of the aetiology and pathogenesis of the disease itself. Rheumatoid arthritis is an example of a chronic proliferative synovitis of diarthroidial joints. Its pathogenesis cannot be readily assigned to an infectious, physical or metabolic stimulus. All other aspects, such as extra-articular manifestations or the presence of rheumatoid factor, are not essential for the existence of rheumatoid arthritis as a defined entity. To date there are no ideal models of rheumatoid arthritis and most other rheumatoid diseases of obscure aetiology. However, there have been several animal models developed that bear some synergy with the human disease. The following is a brief overview of the most relevant models for rheumatoid arthritis.

1.3.1 Adjuvant Arthritis.

One of the most popular models of chronic, immunologically mediated inflammation is the rat adjuvant arthritis (AA), a disease produced by the injection of Freund´s complete adjuvant into certain

dermal and subdermal tissue sites, usually the hind paw (Newbould, 1963). A local reaction develops immediately; a few days later the hind and forepaws swell and arthritic nodules appear on the ears and tail. This secondary reaction is termed "adjuvant disease" and is thought to represent a delayed hypersensitivity response to mycobacterial antigens (Pearson, 1956 and 1979).

AA can only be induced in rats and the increased susceptibility to the disease in certain strains of rats suggests that the condition probably has a genetic basis (Perlik and Zidek, 1973). Selective breeding for high or low responsiveness to adjuvant was used to derive two inbred strains of rats from a Wistar/Sprague-Dawley colony. Wistars were found to be entirely resistant to AA, whilst Sprague-Dawley rats were susceptible - the females affected severely compared to the males. In addition, AA is influenced by the age of the rat. Glenn and Gray (1965) found both young (< 21 days) and old (> 6 months) rats to be relatively resistant to the disease.

As high as 95 - 100% successful induction of the disease is attainable if the adjuvant is properly prepared, ie. by grinding the mycobacteria in a pestle and mortar before preparing the suspension (Swingle, 1974). The intradermal route is commonly used and is

most effective when given in the foot pad or tail (Glenn and Gray, 1965). The volume and concentration of injected material are important. At least 0.1 mg whole bacteria in a volume of 0.05 - 0.1 ml is necessary to induce AA by foot pad or tail or tail administration. Due to their high susceptibility to the adjuvant, female Sprague-Dawley rats in the age range 6 to 8 weeks are chosen for experiments.

Although this animal model for rheumatoid arthritis is not ideal, there are some striking clinical and pathological similarities between AA and RA. These are summarized in Table 4. Other laboratory findings of AA which resemble RA include anaemia, decreased serum iron, decreased rate of incorporation of iron into red cells (Mikolajew et al, 1969) and elevation of erythrocyte sedimentation rate, serum glycoproteins and copper (Gralla and Wiseman, 1968; Weimer et al, 1968). Rheumatoid factor and antinuclear antibodies, detected in RA, however, have not been reported in AA. Depressed levels of hepatic aminopyrine demethylase and of cytochrome P450, observed in RA, have also been reported in AA (Cawthorne et al, 1976) and may be associated with altered drug metabolism by the liver.

Clinical	AA	RA
Acute and recurrent arthritis Peripheral joints	++	++
Chronic deforming arthritis	++	++
Eye lesions	+	+
Progressive and destructive joint disease	++	++
Pathological		
Acute and subacute synovitis	++	++
Primarily mononuclear cell response	++	++
Invasion of bone and joint space by pannus	++	++
Bursitis and tendinitis	++	++
Ankylosis		
Fibrous	++	+
Bony	+	+

\+ = rarely
++ = commonly

Table 4. Comparison of AA and RA (from Pearson, 1979).

1.3.2 Collagen-Induced Arthritis in Rats.

Antibodies to collagen have been detected in the sera and the synovial fluid of rheumatoid patients (Steffen et al, 1974; Huffstutter et al, 1980) suggesting that autoimmunity to collagen may contribute, at least in part, to the disease process. Following these observations, studies by Trentham et al (1977) have shown that immunization of rats with type II collagen (the type found predominantly in cartilage) results in an inflammatory polyarthritis 10 - 16 days after immunization in approximately 40% of the animals. Collagen antibody titres in the sera of rats with polyarthritis were, in general, higher than in the sera of immunized but nonarthritic rats. Thus like human RA, the polyarthritis in the collagen model may be related in some way to collagen autoimmunity. Pathological changes in the rats of collagen-induced arthritis are simmilar to the acute phase of human RA: there is tenosynovitis, with polymorphonuclear leukocytes and mononuclear cells invading the joint space, and erosion of cartilage from the articular side with relative sparing of the bone. There are, however important differences between rheumatoid arthritis and collagen arthritis. Unlike RA, collagen arthritis does ot occur spontaneously, it is not self-perpetuating and invariably subsides several weeks after onset.

Fluctuating remissions and exacerbations do not occur and the hallmark extraarticular lesions of RA (ie. subcutaneous nodules, serositis and vasculitis) are absent. Also, the variety of abnormal antibody responses associated with RA i.e. rheumatoid factors and antinuclear antibodies, have not been demonstrated (Trentham, 1982).

The most susceptible rats to collagen induced arthritis are Wistar rats aged between 4 and 5 weeks, and the expression of the disease is not influenced by the sex of the animal. The incidence and severity of the disease increases as the amount of collagen injected is increased (Trentham et al, 1977).

Although collagen induced arthritis is a versatile system with some advantages over other models of arthritis, it suffers from the inability to produce a consistant response in the majority of test animals. Moreover, the appearance of arthritis in immunized rats has been reported to be affected by psychological stress. Immunized rats exposed to a caged cat developed cell-mediated immune responses and antibodies to collagen, but arthritis appeared in only 3% of animals in the stressed group (Sloboda et al, 1981).

1.3.3. Antigen-Induced Arthritis In Rabbits.

A chronic synovitis, closely resembling human RA with regard to histopathology and chronicity, can be produced in rabbits by an initial systemic immunization followed by injection of the same antigen into a knee joint (Cooke and Jasin, 1972). This method of induction of arthritis is based on earlier observations of Dumonde and Glynn (1962) on fibrin-induced arthritis. The ensuing antigen-induced arthritis affecting the knee required the presence of antigen in the initial immunization, the development of delayed hypersensitivity, and high titre of circulating antibodies to the inducing antigen (Consden et al, 1971). Ovalbumin and bovine serum albumin have been the the most extensively used antigens in this model of chronic inflammation. Besides rabbits, the disease can be induced in guinea-pigs (Dumonde et al, 1977), mice (Brackertz et al, 1977), and chickens (Oates et al, 1973).

The development of the disease is strain-dependent and genetically related (Blackham et al, 1974). The animals are immunized by two subcutaneous injections of the antigen in Freund´s complete adjuvant, and challenged 6 weeks later by intra-articular injection of the antigen into the knee joint, after the existence of delayed hypersensitivity to the antigen had been established by

skin testing. The rabbits produce an arthritic response in the antigen-injected joint. This continues through an acute phase into a chronic phase (115 days after intra-articular injection) as judged by the presence of villous hyperplasia of the synovial membrane, pannus, lymphocyte and plasma cell infiltration and bone and cartilage erosions (Hunneyball et al, 1978).

Antigen induced arthritis provides a model of arthritis that not only closely resembles RA at both the macroscopic and histological levels, but in its pattern of responsiveness to antirheumatic drugs is also very similar to that of RA. However, the use of this model for pharmacological screening is limited due to the size of the animal and the duration of treatment required to demonstrate activity with disease modifying compounds , particularly those of the slow-acting type such as D-Penicillamine.

1.3.4 The Effects of D-Penicillamine in Experimental Models of Arthritis.

D-Penicillamine is neither analgesic nor anti-inflammatoryiin as much as it fails to inhibit acute experimental inflammations that are suppressed by the aspirin-like, non-steroidal anti-inflammatory (NSAID) drugs (Arrigoni-Martelli, 1979). This implies a

lack of influence on pharmacological mediators of inflammation and a quite different mode of action from that of the NSAID´s.

Whereas the adjuvant arthritis model is considered to be successful for the screening of potentially new NSAID´s, slow acting drugs such as penicillamine, levamisole and chloroquine have little effect on the disease. Early studies by Klamer et al (1968) reported a moderate inhibition of AA with the racemate D,L-penicillamine. Later studies by Liyanage and Currey (1972) did not observe any effect when D-penicillamine was administered at the dose 200 mg/kg orally, from a few days prior to the adjuvant injection to 20 days later. Worstmann and Kretzchmar (1975) reported an inhibitory effect with the very high dose of 464 mg/kg. In his experiments, Arrigoni-Martelli (1979) observed that 50 mg/kg of D-penicillamine given to rats with already developed adjuvant arthritis or from the day of adjuvant injection to 28 days later enhanced the severity of the syndrome. No effect was observed when a similar dose was administered for a few days prior to and immediately after the adjuvant injection.

The effect of D-penicillamine in the established model of collagen arthritis is similar to clinical observations in human rheumatoid arthritis. From X-ray analysis, Sloboda et al (1981) showed that rats with

the established lesion of collagen polyarthritis had decreased joint destruction on treatment with a daily oral dose of 200mg/kg D-penicillamine, treatment commencing the same day as the animal was immunized with type II collagen. However, the decrease in joint destruction occurred in the absence of an anti-inflammatory effect. The authors stress the importance of radiographic evaluation of the efficacy of the drugs being studied in this model, a criteria not always acceptable for routine drug screening.

As stated previously, the rabbit model of antigen induced arthritis is probably the most closely correlated model to the human disease. Hunneyball et al (1978) used this model to study the effects of D-penicillamine on the immune response and development of the disease. In these experiments, they managed to show diminution of the arthritis after daily oral administration of D-penicillamine at doses equivalent to that usually administered to rheumatoid patients (ie. 10 - 15 mg/kg body weight). With D-penicillamine treatment beginning 16 weeks after the onset of chronic monoarticular arthritis, the severity of the chronic synovitis was reduced in a considerable proportion of the animals. This was reflected in a reduction in joint circumference beginning 40 - 50 days after initiation of treatment, and this parallels the delay observed in rheumatoid patients between the initiation of treatment

and the onset of effect. When D-penicillamine therapy was initiated prior to immunization, a greater reduction in joint swelling, histopathology and macroscopic score was obtained (Hunneyball et al, 1979).

1.4 SCOPE OF THE STUDY.

The aim of this project was to examine the metabolism of D-penicillamine in an experimental model of arthritis and a healthy control in order to expose any fundamental differences which may help explain the mode of action (therapeutic or toxicological) of the drug. As reviewed in the previous section, all the available animal models have their pros and cons. For metabolic purposes, rat adjuvant arthritis was considered to be the most suitable representation of the disease, having taken into account the practical constraints imposed. It is easily induced, with the arthritis developing to a uniform degree in the majority of the innoculated animals. In addition, the progression of the disease can be monitored without the need for sophisticated techniques. Also, prolonged drug therapy is avoided, due to the relatively short time-course for the disease.

SECTION 2:

MATERIALS AND METHODS

2.1. MATERIALS

Solvents were HPLC grade (Fison's Ltd., Loughborogh, UK). The water used for HPLC was deionised and doubly distilled by means of a Mili-Q deioniser and water still (Milipore S.A. France).

Materials	Supplier
All chemicals, except where stated:	Fison's Ltd. Loughborough, UK
Picric acid; L-cysteine; Amberlite CG120:	British Drug Houses (BDH) Ltd., Poole, UK
Glutathione; 5,5-dithiobis (2-nitrobenzoic acid) (DTNB):	Sigma Chemicals Co. Ltd., Poole, UK
D-(carboxy-^{14}C)-Penicillamine hydrochloride	Lilly Research Centre Windlesham, UK
Penicillamine Disulphide:	Fluka AG, Germany
Penicillamine; Mycobacteria tuberculi:	Lilly Research Centre Windlesham, UK

Creatinine standard:	Beckman Instruments, Fullerton, UK
Spherisorb-NH_3 (5um):	Jones chromatography, Llanbradach, Wales, UK
Scintillation vials: (plastic, 5mls)	Richardsons of Leicester, UK.

2.2 EQUIPMENT

Equipment	**Supplier**
LKB 1215 Rackbeta Scintillation Counter:	Rackbeta, Finland
PU 8610 Spectrophotometer:	Pye-Unicam Ltd., Camridge, UK.
MSE Mistral Centrifuge:	MSE Scientific Instruments, Sussex, UK.
Rat Metabowls:	Jencons Ltd., Hertfordshire, UK.

2.3. SYNTHESIS OF L-CYSTEINE-D-PENICILLAMINE MIXED DISULPHIDE.

The following is an adaptation of the method originally described by Crawhall et al (1964).

Free base L-cysteine (51.43g, 0.48mole) and D-Penicillamine hydrochloride (16.71g, 0.09 mole) were dissolved in ammonium hydroxide (1000 ml, 4 mole) in a Buchner flask, to which ferric chloride (10 ml, 5% w/v in water) was added. Air was drawn through the solution for 24 hours via a wash bottle containing ammonium hydroxide (500 ml, 4N). The precipitated cystine and ferric hydroxide were allowed to settle out from the mixture. The supernatant was removed by suction and evaporated to dryness at 65-70^{o}C (bath temperature). This crude disulphide was washed with cold water (2L) and filtered to remove insoluble material.

Amberlite CG120 (Na) (423g) was converted to the H^{+}form by washing and stirring in a large beaker for a total of 2-3 hours with portions of HCL (1N) (7.5L). After decanting the acid the residual resin was washed free of Cl^{-} with portions of deionized water (4x3L).

The combined crude disulphide washings were added to the damp resin and stirred for 0.5 hour with checking

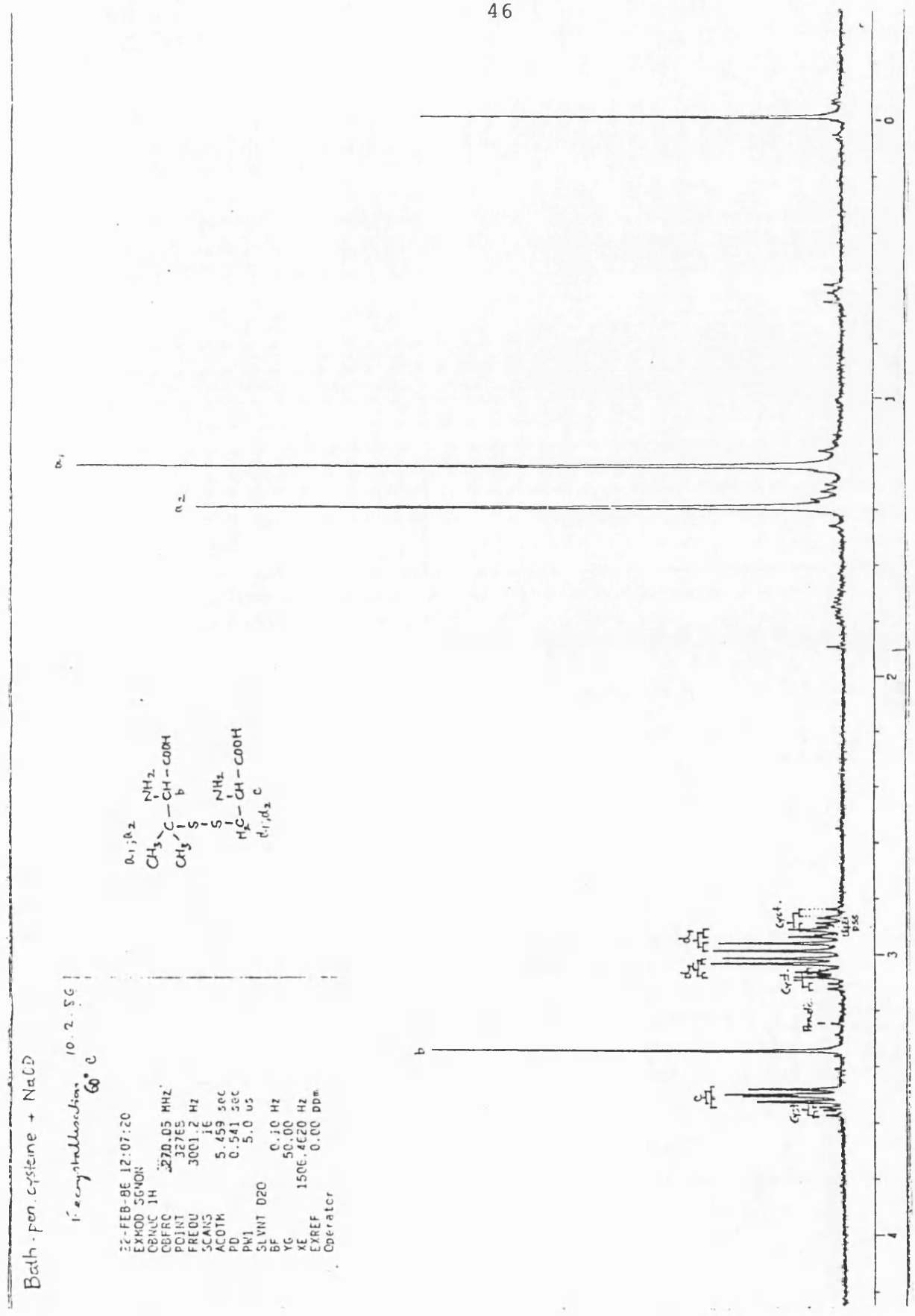


Fig. 2.1. NMR Identification of Penicillamine Cysteine.

for eventual abscence of amino acid from solution (2 drops of solution on TLC plates and 2 drops 5% ninhydrin in EtOH to give a purple colour on heating to 110^{o}C if any amino acid remains). The resin was washed by stirring with water (4x1L) until Cl^- ions were no longer detectable with $AgNO_3$. After decanting the water the resin was stirred with ammonium hydroxide (2N, 4.5L) in 1L portions and the combined filtered eluates evaporated to dryness leaving a light yellow solid (20g) of mixed disulphide as identified by NMR (see Fig. 2.1). Contamination with penicillamine disulphide and cystine was 6.3% and 7.7% respectively.

2.4 ANIMALS

All animals used were female, Sprague-Dawley rats bred in the Bath University Animal House. Weights at the starting date of any experiment were between 120-160g.

2.4.1 Dosing.

All rats that were to be orally dosed were starved the previous night and for 3 hours after dosing to ensure maximal absorption of the drug. Subsequently, they were allowed food and water ad libitum. Drugs for intravenous dosing were made up in 0.9% saline. Drugs for oral dosing were made up in a solution of 5 mls

tween 80 (1%) in 100 mls Carboxy methyl cellulose (0.5%).

2.4.2 Urine Collection.

Urine was collected from animals housed in individual metabowls. Food and water were allowed ad libitum and animals were kept in the metabowls for a maximum of 24 hours.

2.4.3 Serum Collection.

Rats were initially anaesthetized in ether and blood withdrawn by cardiac puncture into vials containing no anticoagulant. Vials were left at room temperature for at least 15 minutes then spun at 3000 rpm for 10 mins. Serum was removed into sterile vials and stored at -20^{o}C until use.

2.4.4 Cannulations.

Rats were anaesthetized with hypnorm/midazolam. ^{14}C-D-penicillamine (10 µCi; 200mg/kg) was introduced into the rat via a cannula in the right jugular vein. Serial blood samples (0.3 ml) were removed via a cannula in the left carotid artery into Eppendorf vials containing EDTA. Blood volume was replaced throughout the experiment with saline. The animal was kept warm by

a thermal blanket maintained at 37°C. Adjuvant arthritic rats were used on Day 15 of the course.

2.4.5 Induction of Arthritis

Polyarthritis was induced by a single injection into the right hind paw of 0.05 mls of a 2.5 mg/ml solution of mycobacterium Tuberculosis in liquid paraffin. The mycobacterium was ground in a pestle and mortar and was stored reground for at least 1 month before use. The day of injection of the adjuvant is designated Day 0.

Rats in the treatment group received the test drug from Day -1 to Day 17. Normal control rats received equivalent volumes of vehicle.

Animals were housed in cages of 3 throughout the adjuvant course.

2.4.6 Evaluation of Arthritis

The following parameters were used to assess the severity of the arthritis:

i) Weight change (Winder et al, 1969). Changes in body weight and retardation in weight gain throughout the 18 day adjuvant course were used to monitor the course of the disease and the response to drug treatment.

ii) Paw volume. Initially right hind paw volume was measured by mercury volume displacement as the paw was dipped as far as the ankle into a small bath of mercury. The displacement of the mercury was monitored by a pressure transducer linked to digital readout calibrated on a linear scale of 1-10. As the disease began to spread, the left hind paw volume was also measured from Day 9 onwards.

iii) Severity of arthritic lesions (Currey and Ziff, 1968). This was based on the degree of joint movement in the four paws. Severity of arthritic lesions was graded using a scoring system of 1 = minor, 2 = moderate, 3 = severe for each paw, thus a maximum score of 12 may be obtained.

2.5. BIOCHEMICAL MEASUREMENTS

2.5.1 Protein Concentration - Folin Assay.

(As described by Lowry et al, 1951).

The following solutions were prepared:

a) 5% w/v $CuSO_4.5H_2O$

b) 10% W/v NaK Tartrate

c) 2% w/v Na_2CO_3 in O.1M NaOH

d) 1 vol. Folin - Ciocalteau reagent plus 2 vols. distilled water

e) 1 ml of a) + 1 ml b) + 8 ml H_2O

f) 1 ml of e) + 50 ml c)

Solutions e) and f) were prepared immediately before use.

Incubations were set up in duplicate. 0.4 ml sample was added to 4.0 ml solution f) and 0.4 ml solution d). Tubes were whirlimixed and left to stand for 30 mins in the dark at room temperature. The absorbance at 650 nm was read against a reagent blank. Protein concentrations were calculated from a calibration curve prepared using bovine serum albumin in saline as standard to a maximum concentration of 400 ug/ml. Samples with a protein concentration higher than this were diluted as appropriate with saline.

2.5.2 Urinary Creatinine Concentrations

The following stock solutions were prepared:

a) 35mM Picric acid

b) 1.6M NaOH

c) 1.2M TCA

Urine samples were diluted 1 in 50 with distilled H_2O. 0.5 ml of diluted sample was pipetted into a test tube

to which 0.5 ml TCA solution and 1 ml picric acid : NaOH (1:1) solution was added. The tubes were whirlimixed and incubated for 20 mins in a water bath maintained at 25°C. Absorbance was measured against a water blank, treated as above, at 490 nm with the spectrophotometer block maintained at 25°C. Creatinine concentrations were calculated from a calibration curve prepared from creatinine standards in the range 0.1 to 5.0 mg/dl.

2.5.3 Serum and Urinary Copper Concentrations

Serum and urine samples were diluted 1 in 10 with deionized, double distilled water. Copper levels were measured on an SP9 Automatic Absorption Spectrophotometer, previously calibrated with solutions of known copper concentrations.

2.5.4 Serum and Urinary Sulphydryl Levels.

The following stock solutions were prepared:

a) 0.1M phosphate buffer pH 7.4
b) 0.8 mg/ml DTNB in 0.1M phosphate buffer made up immediately before use and stored in a dark brown bottle.

c) 1mM glutathione in 0.1M phosphate buffer.

i) Serum.

0.05 ml serum was pipetted into a 1 ml cuvette to which 0.75 ml 0.1M phosphate buffer and 0.2 ml DTNB were added. Cuvettes were whirlimixed and incubated for 5 mins. at 37°C. Absorbance was measured at 440 nm against a blank of 0.05 ml serum plus 0.95 ml 0.1M phosphate buffer with the spectrophotometer block heater maintained at 37°C.

ii) Urine.

0.5 ml urine was pipetted into a 1 ml cuvette to which 0.3 ml 0.1M phosphate buffer and 0.2 ml DTNB were added. Cuvettes were whirlimixed and incubated for 5 mins at 37°C. Absorbance was measured at 440 nm against a blank of 0.5 ml urine plus 0.5 ml 0.1M phosphate buffer with the spectrophotometer block heater maintained at 37°C.

Calibration curves were constructed using glutathione solutions of known concentrations (not exceeding 1mM). Absorbance of standards (treated as for serum samples) were read against a blank of 0.05 ml 1mM glutathione plus 0.95 ml 0.1M phosphate buffer.

When calculating urinary sulphydryl levels a multiplication factor of 0.1 must be used to account for the ten-fold increase in sample volume to standard volume.

2.6. THIN LAYER CHROMATOGRAPHY

The following system was employed to separate and identify penicillamine, Penicillamine disulphide and penicillamine cysteine disulphide.

Mobile phase:

90% Methanol
7% Water, deionized,double distilled
3% 6M Acetic acid

Stationary phase:

TLC aluminium sheets coated with silica gel 60 (without fluorescent indicator), 0.2 mm thick (E.Merck, Darmstadt, Germany

Amino Acid Visualisation:

0.3g ninhydrin in 100 ml butan-1-ol plus
3 ml glacial acetic acid.

After running in the above mobile phase, TLC plates were completely dried, then sprayed with the ninhydrin solution and heated to 110^{o}C in a small oven. Amino acids appeared as small purple spots from which Rf values were calculated.

In radiolabelled-TLC studies, TLC plates were divided into 0.5 cm strips horizontally, and the silica gel scraped off into seperate insert vials to which 4.5 mls Optiphase scintillation fluid was added. Vials were whirlimixed and radioactivity measured.

2.7 D-PENICILLAMINE DETERMINATION BY HPLC.

The measurement of D-Penicillamine by HPLC employed an elaborate two column system coupled to a gold-mercury electro-chemical detector. This was a modification of the method initially described by Abounassif and Jefferies (1983). This system allowed unwanted interfering plasma peaks, the majority of which elute before the penicillamine peak, to be diverted away from

the detector but allowed the penicillamine to pass to the detector. Thus, this protected the sensitive electrode from "coating" by plasma compounds and prevented a loss in electrode sensitivity.

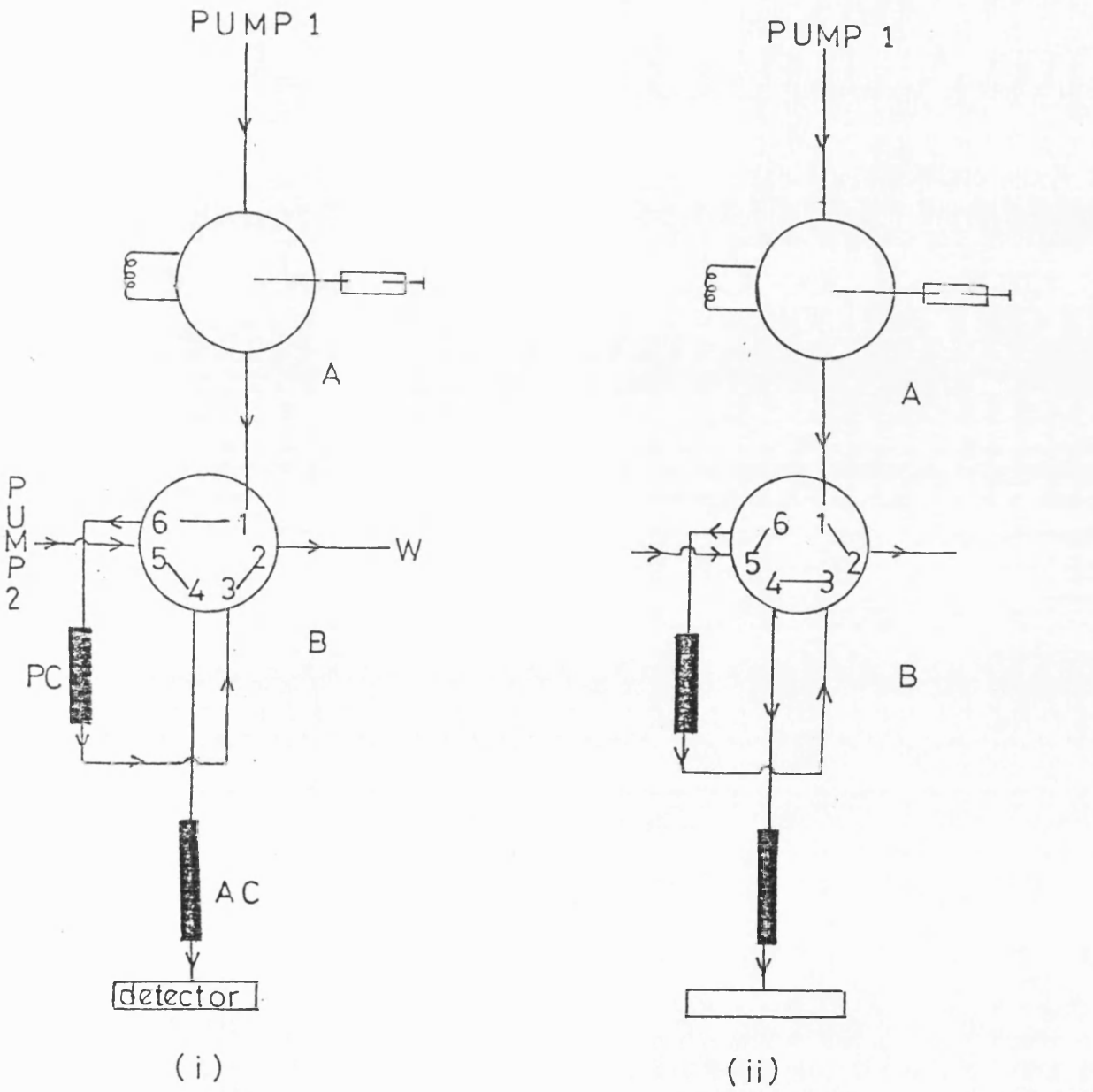
The overall configuration of the system is shematically represented in Fig. 2.2.

HPLC Equipment

Constant flow pumps (Consta Metric III pumps, L.D.C. Ltd., Stone, Staffs, UK) were fitted with additional custom-made pulse dampers to maximise a pulse-free solvent flow. Samples were introduced by means of a high-pressure injection valve fitted with a constant volume loop (0.1 ml) (Rheodyne 7125, Rheodyne Ltd., Cotati, California, USA). All column fittings, tubing and plumbing were supplied by HETP Ltd., Macclesfield, UK.

Switching of the solvent flow was achieved by means of a high-pressure switching valve (Rheodyne 7000, Rheodyne Ltd.)

The column system was maintained at 37^{o}C by a heated water bath (Type 400.010, Gallenkamp, Loughborough, Leics. UK).



A = Rheodyne injection valve
B = Rheodyne switching valve
PC = pre-column
AC = analytical column
W = waste

Fig. 2.2. Column arrangement and port positions for switching system.
(i) Loading pre-column and flushing to waste
(ii) Transfer from pre-column to analytical column.

The column eluent was monitored by passing it through the flow-cell of an electrochemical detector (BAS LC-4A, Bioanalytical systems, West Lafayette, USA) fitted with a thin-layer cell containing a gold-mercury electrode (BAS, TL-6A). The reference electrode was an Ag/AgCl electrode (RE-1).

The detector output was recorded on a potentiometric chart recorder (JJ CR650S recorder, JJ Ltd., Southampton, UK).

Mobile Phase

HPLC grade methanol and deionized water were used to make up mobile phases to minimize contamination. The mobile phase used in all systems for measuring penicillamine in biological fluids consisted of 0.05M citrate buffer (pH 3.2), 4% (v/v) methanol, 5×10^{-3}M sodium dodecyl sulphate and 0.1 g/l EDTA. All mobile phases were freshly prepared and filtered using 0.45 um filters (Type HA, Micropore filters, Millipore Ltd., Harrow, Middlesex, U.K.) Mobile phase pH was checked using a Howe 6031 pH meter, V.A. Howe Ltd, London, UK).

After filtration mobile phases were degassed under vacuum exceeding 500 mmHg for 20 mins. The degassed condition was maintained by a Du-Pont flotation

degassing system which is effective for at least 24 hours.

2.7.1 Total D-Penicillamine in Serum.

Calibration Curve.

Stock solutions of D-Penicillamine in mobile phase containing 1 g/L EDTA were used to prepare standards in serum in the range 5 mg/L (0.335×10^{-4}M) to 250 mg/L (1.667×10^{-3}M). 0.1 ml aliquots of the appropriate D-penicillamine solution in mobile phase were added into aliquots of 0.9 mls. blank serum. The "spiked" serum samples were then treated as for test samples as described below. Calibration curves (Fig. 2.3) were repeated before each assay batch along with a 10 µg/ml D-penicillamine standard throughout the assay. The coefficient of variation was less than 3% throughout the day.

Sample Treatment.

1 ml of sample/standard was pipetted into glass flow tubes to which 0.72 ml of dithiothreitol (20 mg/ml) was added, followed by 1.28 ml of disodium hydrogen phosphate buffer (0.05M) to give a final volume of 3 mls. The tubes were thoroughly whirlimixed and

incubated in a water bath maintained at 60°C for 3 hours. Upon removal from the water bath, the tubes were again thoroughly whirlimixed and the tubes then spun at 4,500 rpm for 25 mins. in a Hettich Universal Centrifuge (Arthur Horwell ltd., London U.K.). The supernatants were removed and pippetted into microfuge tubes (Sterilin Ltd., Feltham, Middlesex, U.K.) which were then spun in an Eppendorf microfuge at maximum speed for 1 minute to further remove any debris. The supernatants were then filtered through Amicon microfilters (0.2 um pore size, type YMT, Amicon Ltd., Stonehouse, Gloustershire, U.K.). The filtrates were then added to an equal volume of mobile phase containing 1 g/L EDTA in small sample tubes (TSP1/2, Fisons Ltd., Loughborough, UK). 0.1 ml of sample was injected onto a precolumn (15 cm Spherisorb-NH_3, 5 um, pump flow rate 1.45 ml/min.) whose outlet flushed to waste, by means of the injection valve. At 7.5 minutes precisely, the running sample was transferred to the main analytical (5 cm, Spherisorb-NH_3, 5 um, pump flow rate 1.35 ml/min.) by means of the swithching valve. The eluate then flowed directly to the detector whose electrode was maintained an an applied potential of 0.15V. After elution of the penicillamine peak the flow was switched back using this valve so that the next sample could be introduced onto the precolumn. All samples were maintained at 4°C before measurement and were injected in duplicate, in the order standard,

sample no.1 (duplicated), sample no.2 (duplicated), standard, and so on. Concentrations of total D-Penicillamine in unknown serum samples were calculated from the standard curve. A typical chromatogram from the sera of D-penicillamine treated rats, showing the injection order is illustrated in Fig. 2.4.

2.7.2 Total D-Penicillamine in Urine.

Determination of total D-Penicillamine in urine was performed as above except on a one-tenth scale, ie incubations consisted of 0.1 ml sample (or standard), 0.072 ml dithiothreitol (20 mg/ml) plus 0.128 ml disodium hydrogen phosphate buffer (0.05M) to give a final volume of 0.3 ml. After the three hour incubation at 60^{o}C, tubes were removed straight onto ice and 0.2 ml of mobile phase containing 1 g/L EDTA was added. Sample injections and valve switching times were as above. Fig. 2.5 shows a representative calibration curve.

2.7.3 Free D-Penicillamine in Serum

Blood for free D-penicillamine determinations was collected into EDTA vials. After centrifugation at 3000

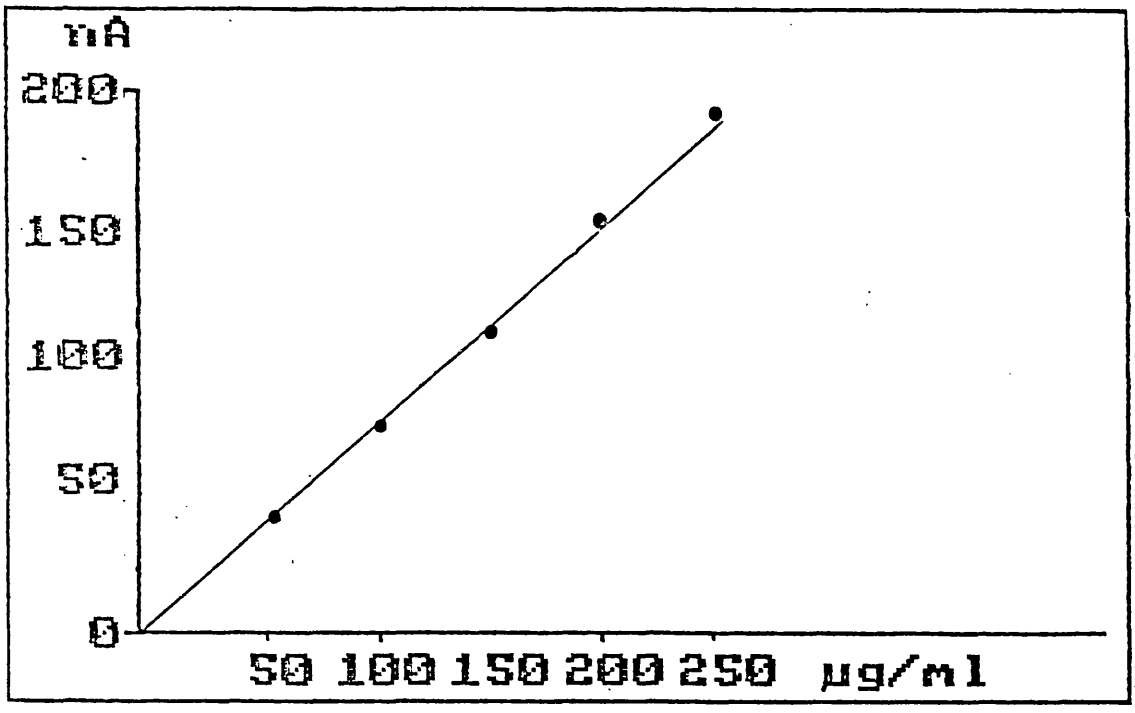


Fig. 2.3. Calibration curve for total D-penicillamine in rat serum. ($r > 0.99$)

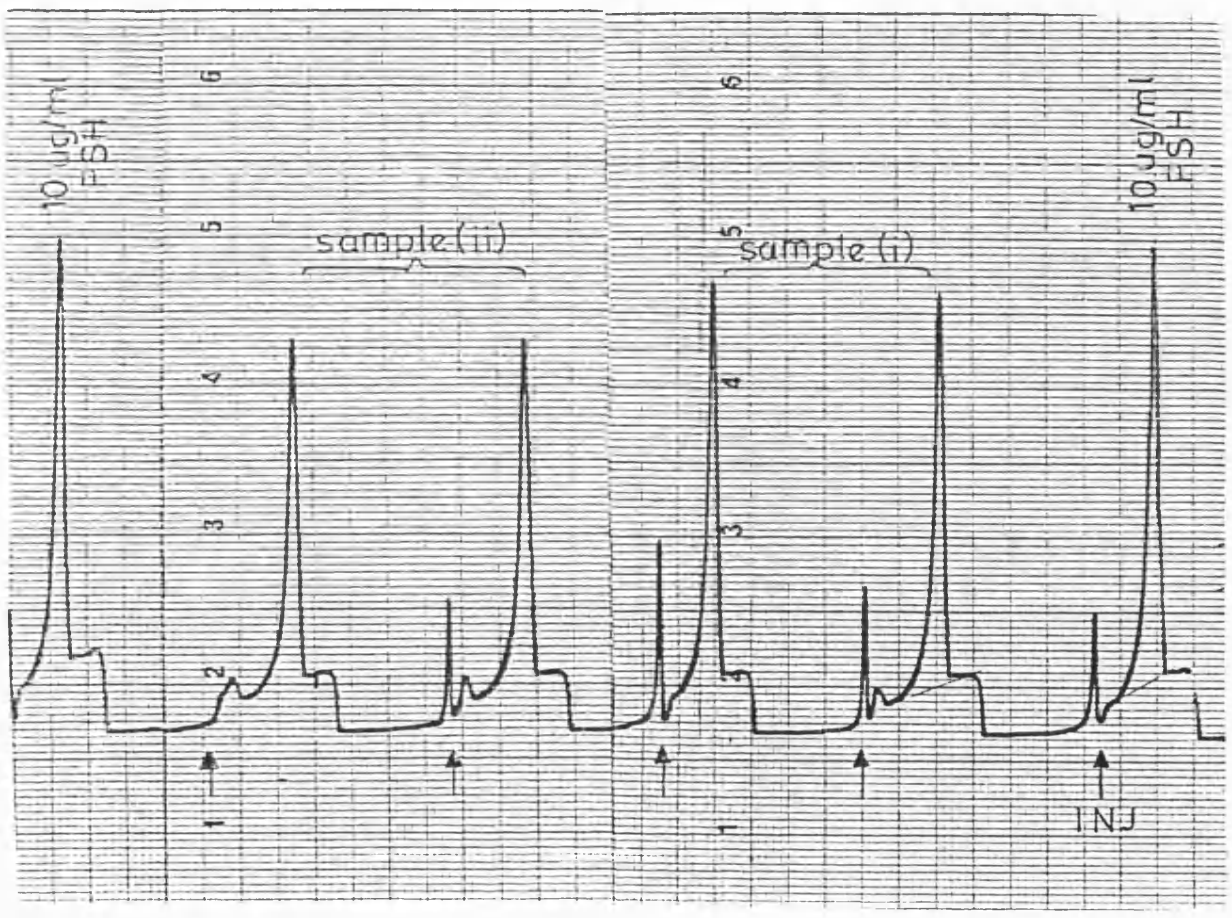


Fig. 2.4 Typical chromatogram for total D-penicillamine in rat serum. Spiked standard samples corresponding to 10 µg/ml are also shown.

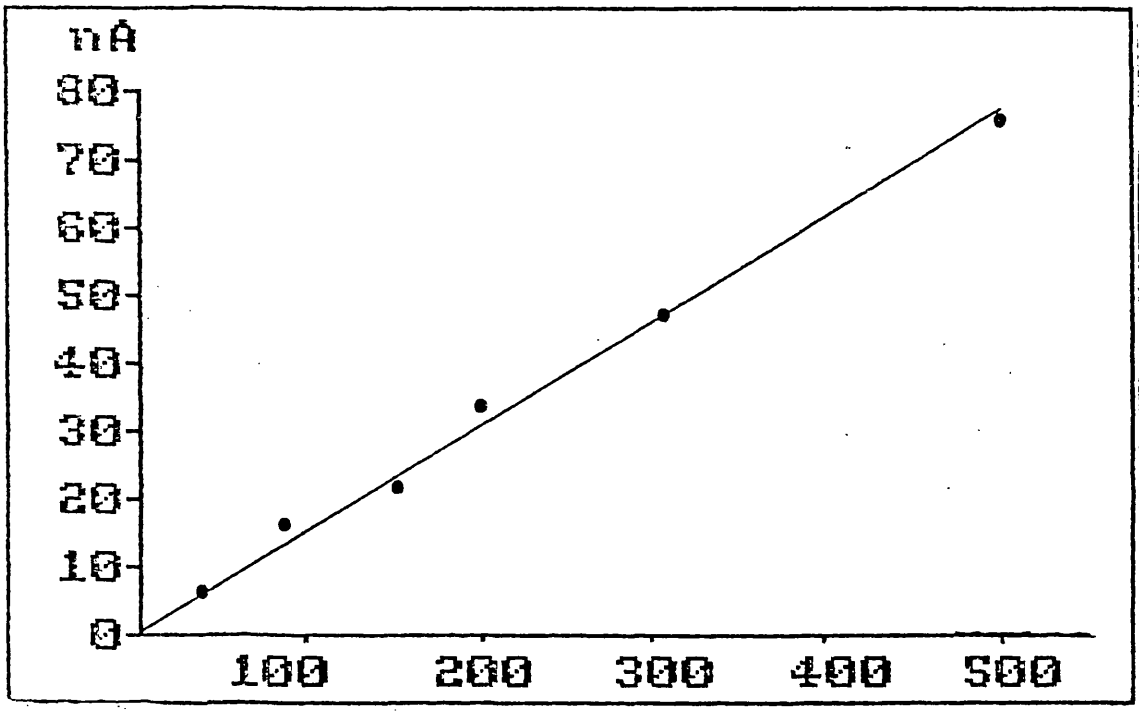


Fig. 2.5. Calibration curve for total D-penicillamine in rat urine. ($r > 0.99$)

rpm for 10 mins. the supernatant (1 ml) was transferred to an Amicon filtration unit. Units were spun for 25 mins. at 4,500 rpm. The resulting ultrafiltrate was then mixed 1:1 with mobile phase (containing 1 g/L EDTA) and maintained at $4^{o}C$ until assayed as above. Calibration curves were prepared from "spiked" ultrafiltrate.

Statistical Analysis

The Student´s "t" test was routinely used as the data was assumed to be normally distributed. The level of significance was set at 0.05.

SECTION 3:

RESULTS AND DISCUSSION

3.1 ESTABLISHMENT OF A NON-TOXIC DOSE OF D-PENICILLAMINE

In recent years there have been a number of reports suggesting a genetic predisposition to toxic reactions induced by D-penicillamine therapy in humans (Panayi, 1978; Wooley 1980, Perrier, 1985). Similar studies in rats have highlighted strain variabilities in toxicity to the drug. Donker and co-workers (Donker et al 1984) showed that whilst Lewis and Sprague-Dawley rats, weighing between 120-150g, tolerated a daily oral dose of 20 or 50 mgs of d-penicillamine for 5 months, Brown Norway rats developed severe side effects after 3-8 weeks at the same dose. Subsequent experiments required the administration of D-penicillamine to female Sprague Dawley rats acutely and throughout the period of an adjuvant course. Therefore preliminary experiments were performed to establish a dose of D-penicillamine that could be admnistered both acutely and daily throughout the adjuvant time course, without inducing toxic side effects. A range of biochemical parameters likely to be affected by D-penicillamine were therefore monitored to assess potential toxicological effects.

Proteinuria is one of the most commonly observed side effects of D-penicillamine treatment in man, presumably caused by renal damage. Urinary protein was therefore measured as an indicator of the efficiency of

glomerular filtration and renal damage. However as disturbances in glomerular filtration are not always accompanied by proteinuria (Rickers et al, 1980), urinary creatinine excretion was also monitored. Assuming there is no change in plasma creatinine, an increase of urinary creatinine would be an indicator of renal function.

Serum sulphydryl levels are known to be reduced in both rheumatoid arthritis and adjuvant arthritis (Lorber et al, 1964; Butler et al, 1969). Given the capacity of D-penicillamine to take part in disulphide exchange reactions gross changes in serum and urinary sulphydryls would be expected to reflect any acute toxicological effects of D-penicillamine.

Metal chelation by D-penicillamine, particularly copper, could also result in toxicological manifestations such as neutropenia. As copper is extensively chelated by D-penicillamine copper levels in serum and urine were monitored to ensure that exhaustive depletion did not occur. Increases in copper excretion also served to verify that D-penicillamine at the doses employed was "pharmacologically" active.

In addition to the above parameters, changes in the body weight of the animals was monitored as this is

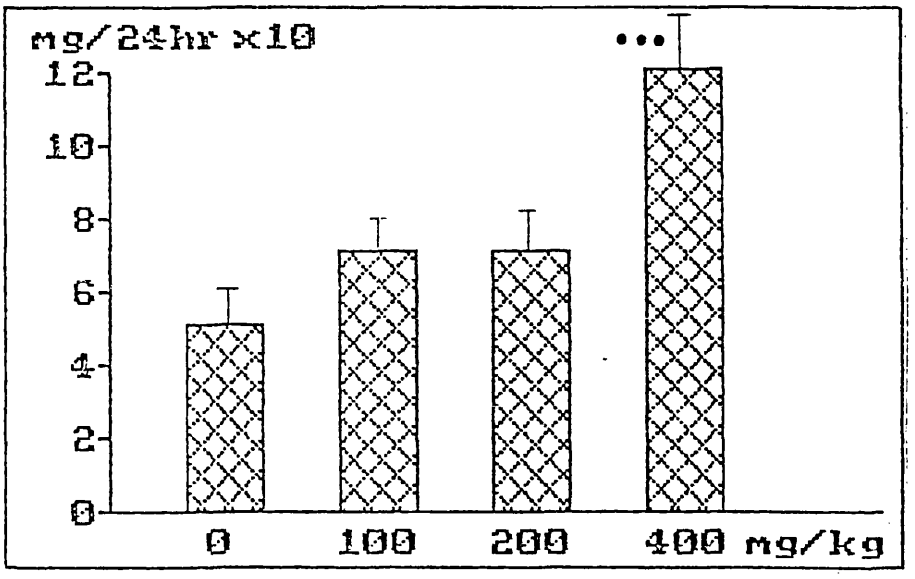
often a useful reflection of the well-being of the animal.

3.1.1. Acute Dosing with D-Penicillamine.

Preliminary experiments involved acute oral dosing of D-penicillamne at three selected doses. Biochemical parameters were compared with controls receiving vehicle only. Female Sprague-Dawley rats weighing between 120-160g were treated with a single, oral dose of D-penicillamine at dose levels of 100, 200 and 400 mg/kg (4 rats per dosage group). Urine was collected over the subsequent 24 hours, from which urinary protein, creatinine, sulphydryl and copper excretions were measured. Blood was also collected at the end of the 24 hour period and assayed for serum copper and sulphydryls by the methods described in Section 2.5.

Figs 3.1.1 (a) and (b) show the effect of a single oral dose of 100, 200 or 400 mg/kg of D-penicillamine on urinary excretion of protein and creatinine respectively, over the subsequent 24 hours post-dosing. Whilst the two lower doses showed no significant difference from controls given only the vehicle, animals treated with 400mg/kg D-penicillamine showed a significant increase in protein excretion, 121.9 $\pm$ 13.8 mg/24 hr as compared to 48.4 $\pm$ 4.9 mg/24 hr for control rats ($p<0.01$). Similarly, urinary creatinine was

(a)



••• $p < 0.01$

•• $p < 0.02$

(b)

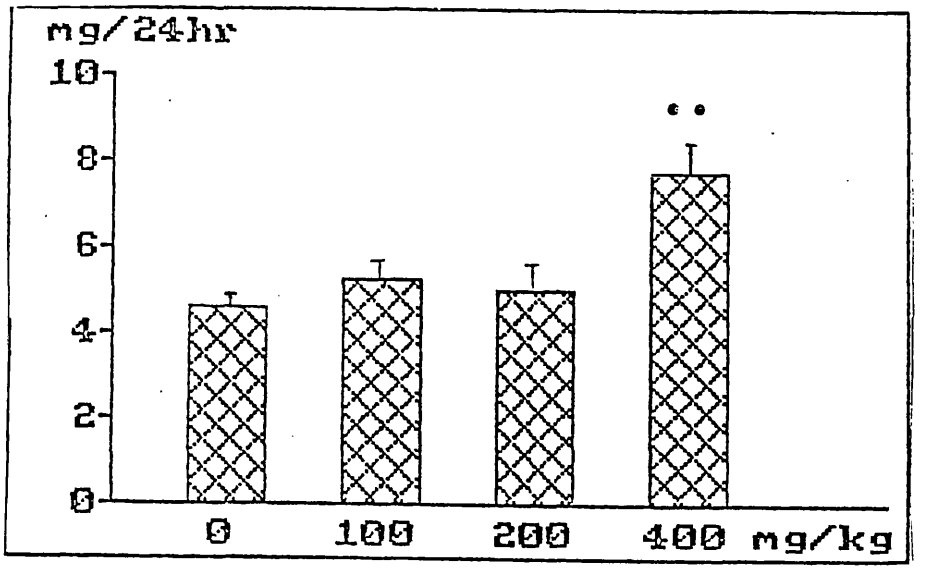


Fig. 3.1.1. Excretion of (a) Protein and (b) Creatinine in rat urine in the 24 hours after a single oral dose of 100, 200 or 400 mg/kg D-Penicillamine (n=4, mean ± S.E.M.)

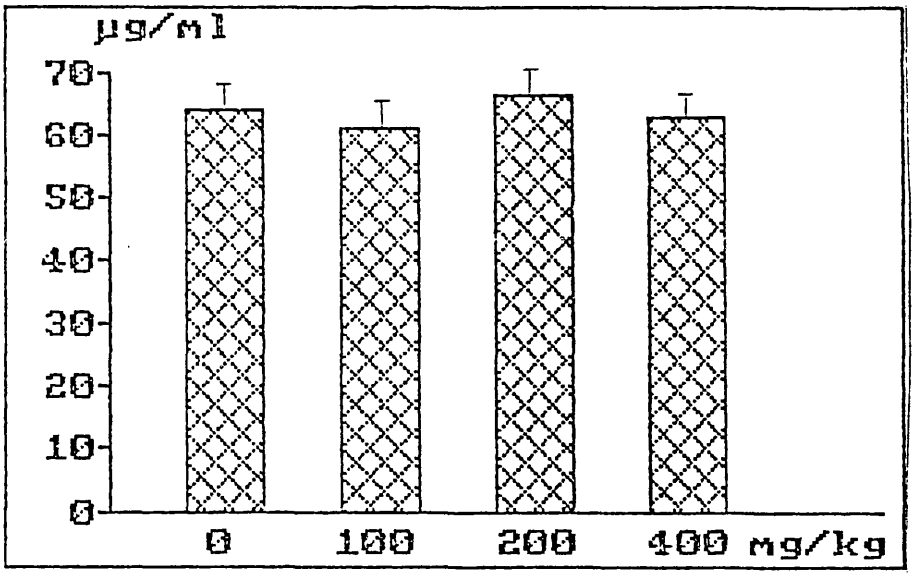
significantly increased at the 400 mg/kg dose with excretion being 7.6 ± 0.8 mg/24 hr, compared to 4.3 ± 0.2 mg/24 hr ($p<0.02$) in vehicle only animals.

As shown in Fig. 3.1.2 (a) serum sulphydryl levels were not significantly ($p>0.05$) affected by D-penicillamine at any dose studied, whereas a dose related increase in urinary sulphydryl excretion (Fig. 3.1.2 (b)) was apparent. A dose of 200 mg/kg D-penicillamine resulted in urinary SH levels of 57.0 ± 1.2 µg/24 hr compared to 41.1 ± 4.8 µg/24 hr in control animals ($p<0.05$). Urinary SH excretion was 83.2 ± 6.7 mg/24 hr after an oral dose of 400 mg/kg D-penicillamine ($p<0.02$).

Increasing doses of D-penicillamine caused a corresponding dose related significant increase in urinary copper excretion, as shown in Fig. 3.1.3 (b). Whilst control values were only 6.5 ± 0.7 µg of copper excreted in 24 hours, doses of 200 mg/kg increased output to 20.4 ± 2.1 µg/ 24 hr ($p<0.01$). Urinary copper excretion was 34.1 ± 4.8 µg/24 hr after an oral dose of 400 mg/kg D-penicillamine ($p<0.001$). However, Fig. 3.1.2 (a) shows that serum copper levels were not significantly different from the control value of 0.98 ± 0.1 µg/ml at any of the three dosage levels.

These results indicate that at a dose of 200 mg/kg the toxic effects of D-penicillamine are not apparent from

(a)



•• $p < 0{\cdot}02$

• $p < 0{\cdot}05$

(b)

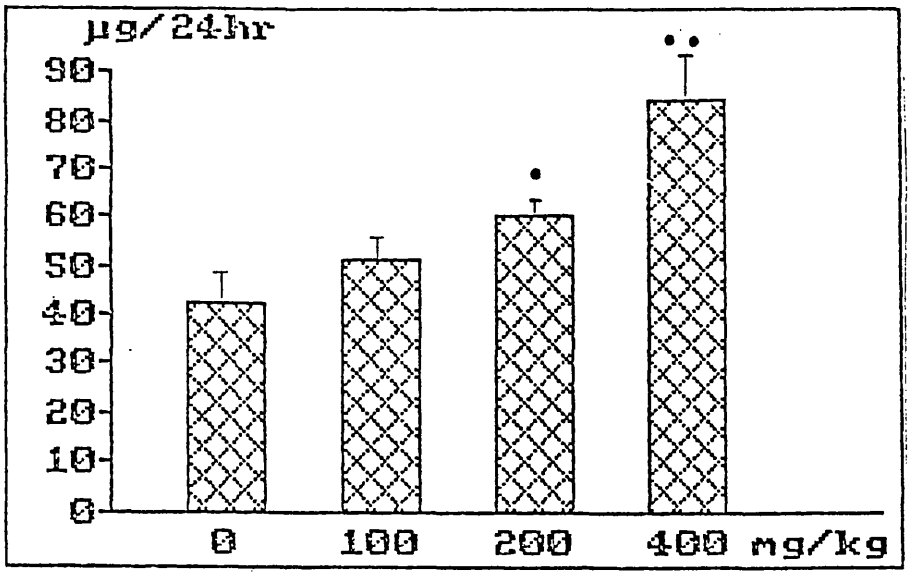
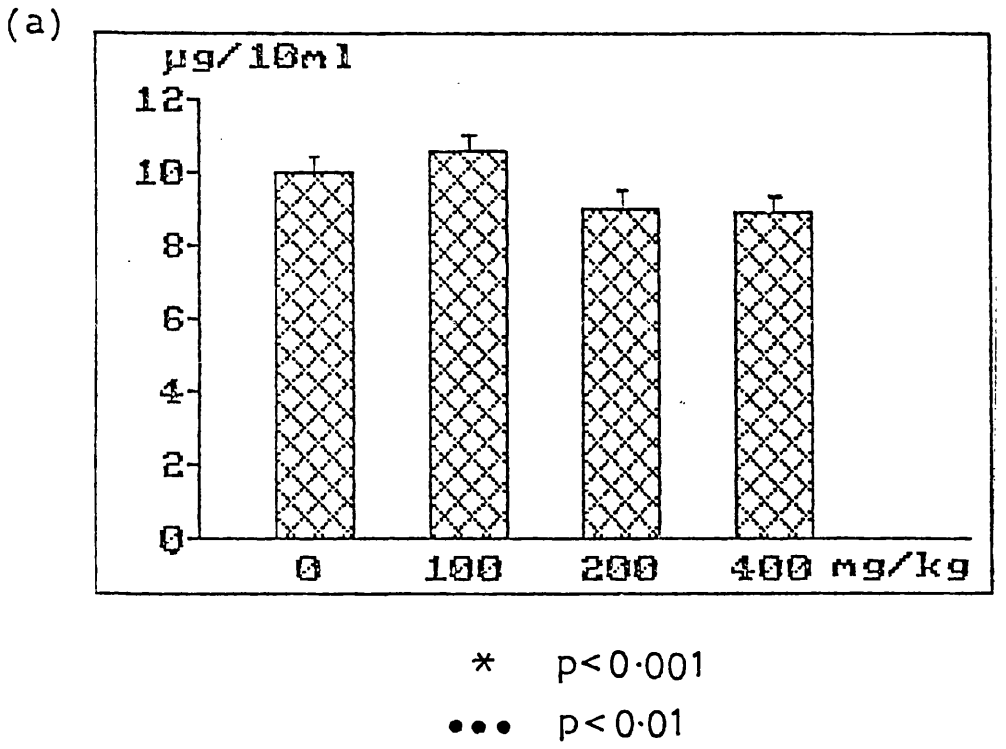


Fig. 3.1.2. Sulphydryl Levels in (a) serum (b) urine in the 24 hours after a single oral dose of 100, 200 or 400 mg/kg D-Penicillamine (n=4, mean ± S.E.M.)



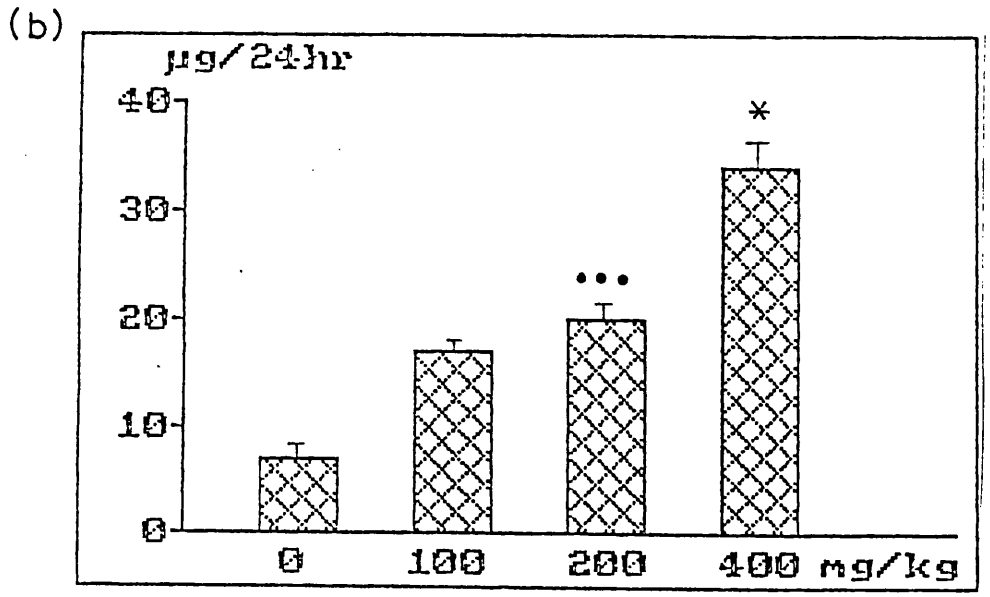


Fig. 3.1.3. Copper levels in (a) serum (b) urine in the 24 hours after a single oral dose of 100, 200 or 400 mg/kg D-Penicillamine (n=4, mean ± S.E.M.)

the parameters monitored, whereas at 400 mg/kg toxic effects are observed. This is particuarly manifest in values for urinary protein and creatinine excretion (Figs. 3.1.1 (a) and (b)) with only the highest dose significantly affecting these parameters, suggesting an immediate interference with renal function at this dose.

From these results it would seem that the maximum "safe" dose of D-penicillamine that can be administered without toxic effects is 200 mg/kg.

3.1.2 Chronic Dosing with D-Penicillamine.

Having established that an acute dose of 200 mg/kg of D-penicillamine could be tolerated with no apparent side effects, the next step was to ensure that chronic treatment with this dose of the drug could also be tolerated with no untoward effects. D-penicillamine was administered orally at a dose of 200 mg/kg for 18 days. The same biochemical parameters as measured previously were monitored on days 1, 3, 7, 9, 15 and 17 after dosing commenced (4 rats per day group). This time period covers the length of the adjuvant course employed.

Figs 3.1.4 (a) and (b) show the effect of the selected dose on urinary protein and creatinine excretion over

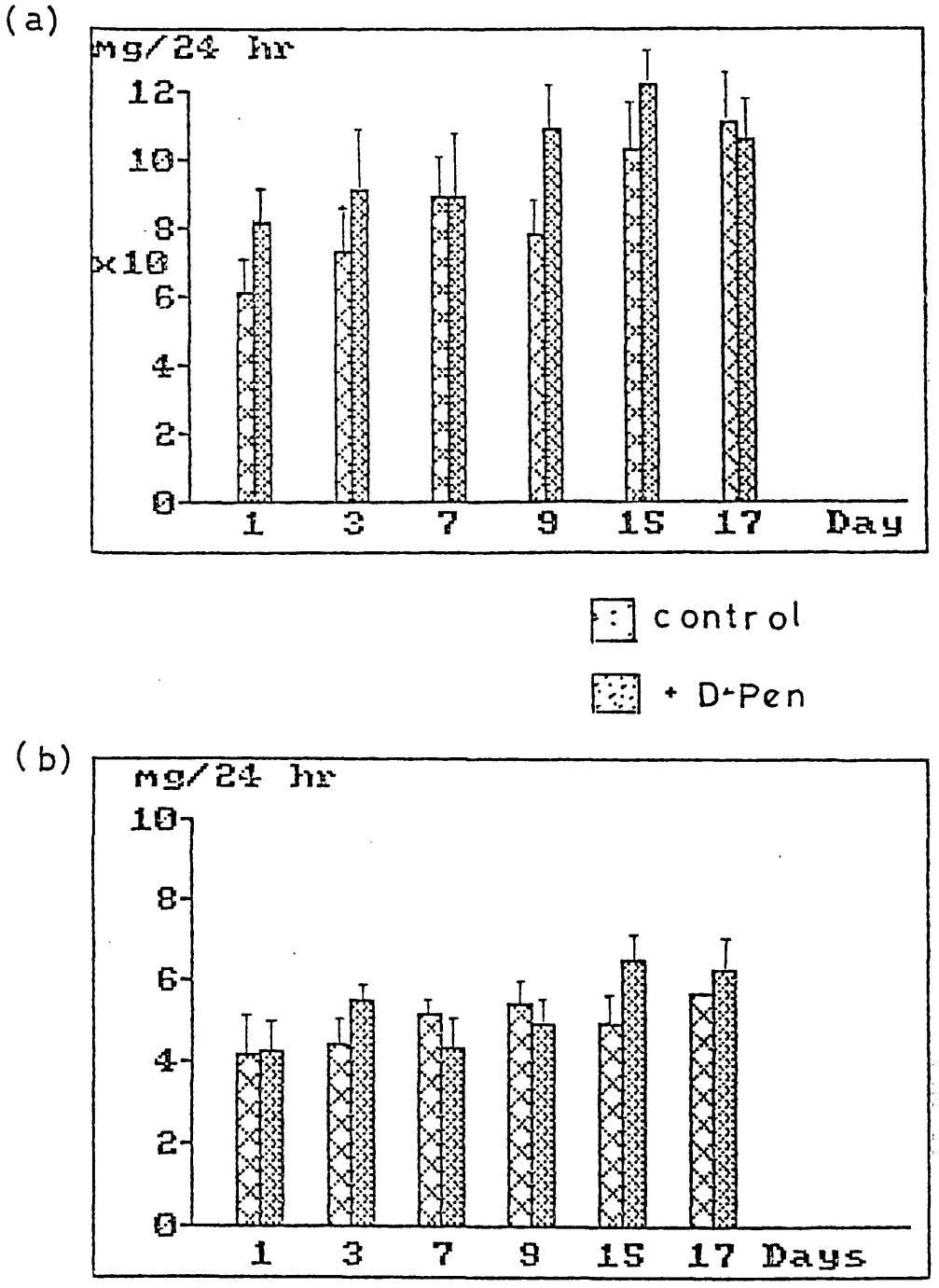


Fig. 3.1.4. Urinary excretion profile of (a) protein and (b) creatinine from control rats and rats treated daily from day 0 with D-penicillamine (200mg/kg p.o) (n = 4 ± S.E.M.)

the 18 day period. Urinary protein increased from 62.0 ± 10.1 mg/24 hr to 104.9 ± 12.6 mg/24 hr in control rats over the 18 day time course as the animals grew and matured. This pattern was not significantly altered by chronic D-penicillamine treatment (200 mg/kg), urinary protein increasing from 80.2 ± 11.2 mg/24 hr on day 1 to 102.3 ± 10.2 mg/24 hr at the end of the course. Urinary creatinine levels increased from 4.1 ± 1.1 mg/24 hr to 5.7 ± 0.5 mg/24 hr in control rats during the 18 days. This was paralleled by an increase (not significantly different from control) from 4.2 ± 0.9 mg/24 hr to 6.1 ± 0.6 mg/24 hr in the rats treated with 200 mg/kg D-penicillamine.

Fig 3.1.5 (a) shows that serum sulphydryl levels did not significantly change throughout the 18 days remaining at around 50 µg/ml. D-penicillamine treated rats were not significantly different from controls during the 18 days. Fig 3.1.5 (b) shows that urinary SH excretion was significantly higher after the first day of D-penicillamine treatment, 41.2 ± 4.6 µg/24 hr in control rats, compared to 64.0 ±4.8 µg/24 hr in the animals dosed with 200 mg/kg of the drug ($p<0.05$). However, on repeated daily dosing with D-penicillamine, total free SH goups in the urine showed no significant difference between the two groups.

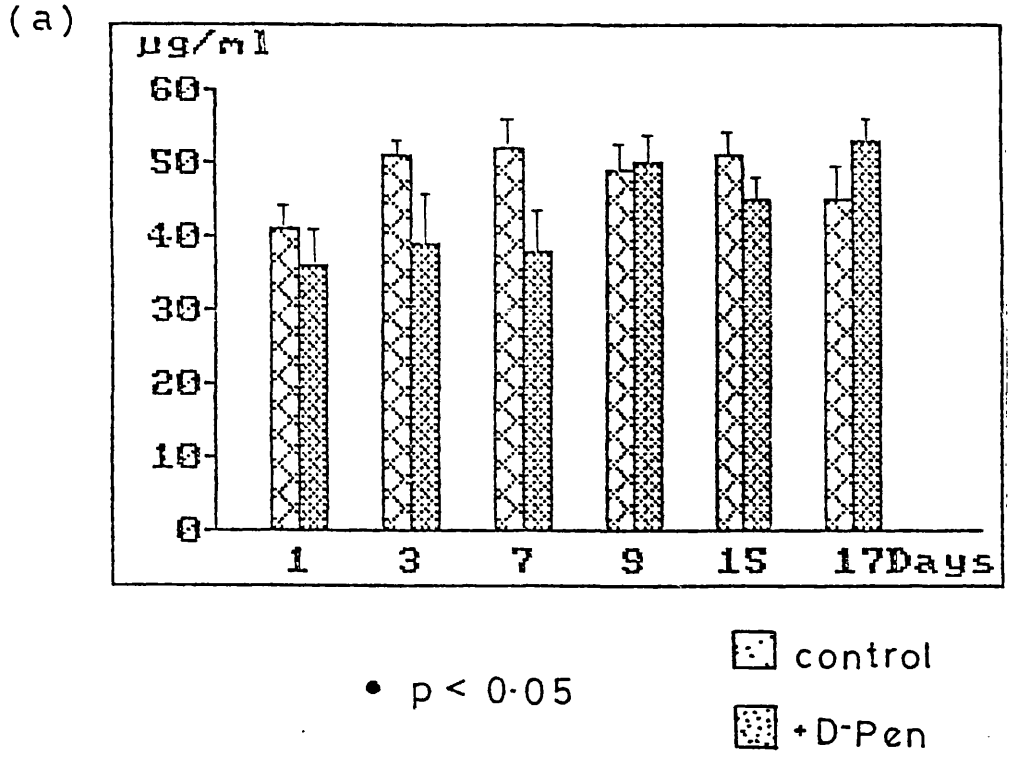


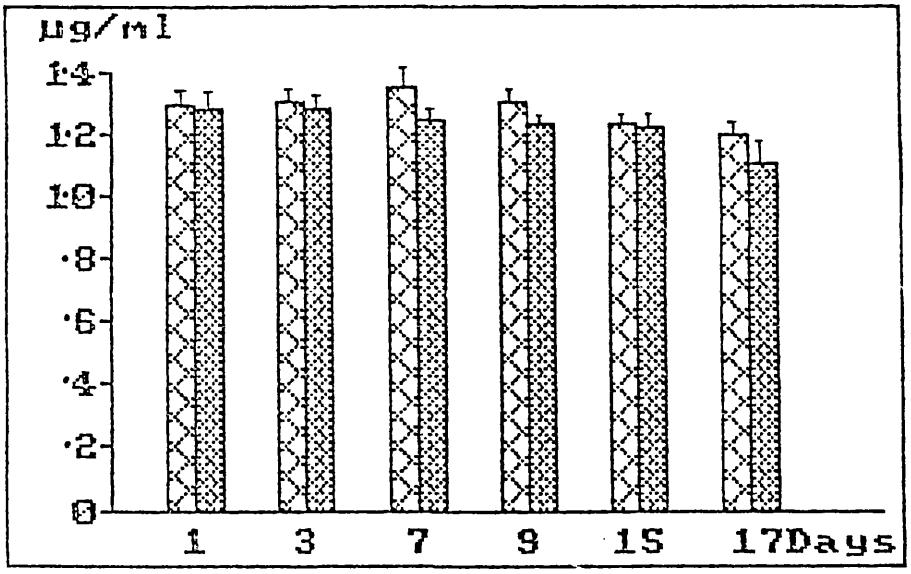
Fig. 3.1.5. Sulphydryl levels in (a) serum and (b) urine from control rats and rats treated daily from day 0 with D-penicillamine (200mg/kg p.o) (n = 4 ± S.E.M.)

The most drammatic effects of the drug were on the urinary copper profile (Fig 3.1.6 (b)). Copper excretion increased rapidly from a control value of 5.0 $\pm$ 0.3 µg/24 hr to a maximum of 30.4 $\pm$ 4.9 µg/24 hr at day 9 ($p<0.001$). Thereafter urinary copper excretion declined to 19.5 $\pm$ 1.8 µg/24 hr on day 17, but this was still significantly higher than control values ($p<0.001$). Serum copper levels however (Fig. 3.1.6 (a)), were unaffected by the drug treatment during the 18 days, remaining between 1.1-1.2 µg/ml in both groups.

As Fig 3.1.7 shows, both control and D-penicillamine treated rats grew rapidly during the 18 days, increasing in weight by 71.2 $\pm$ 1.2g and 72.4 $\pm$ 1.8g respectively ($p>0.05$).

The present results thus show that a dose of 200mg/kg D-penicillamine can be safely administered on a daily oral basis to young, female Sprague-Dawley rats over an 18 day period with no apparent adverse effects. Having established this, a preliminary examination of the kinetics of an acute and chronic dose of the drug was performed in order to correlate the plasma D-penicillamine levels obtained in rats with those in man.

(a)



* p < 0·001

control

+ D-Pen

(b)

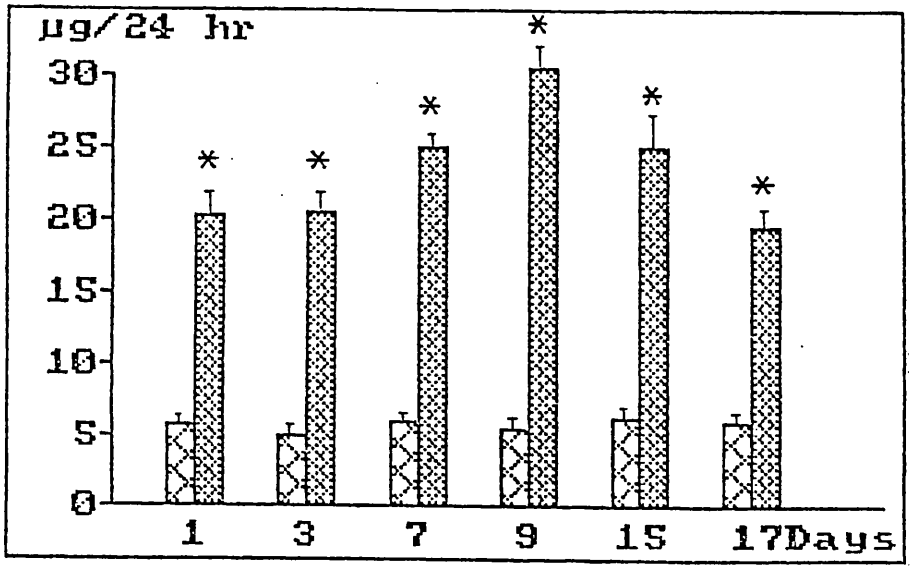


Fig. 3.1.6. Copper levels in (a) serum and (b) urine from control rats and rats treated daily from day 0 with D-penicillamine (200mg/kg p.o). (n = 4 ± S.E.M.)

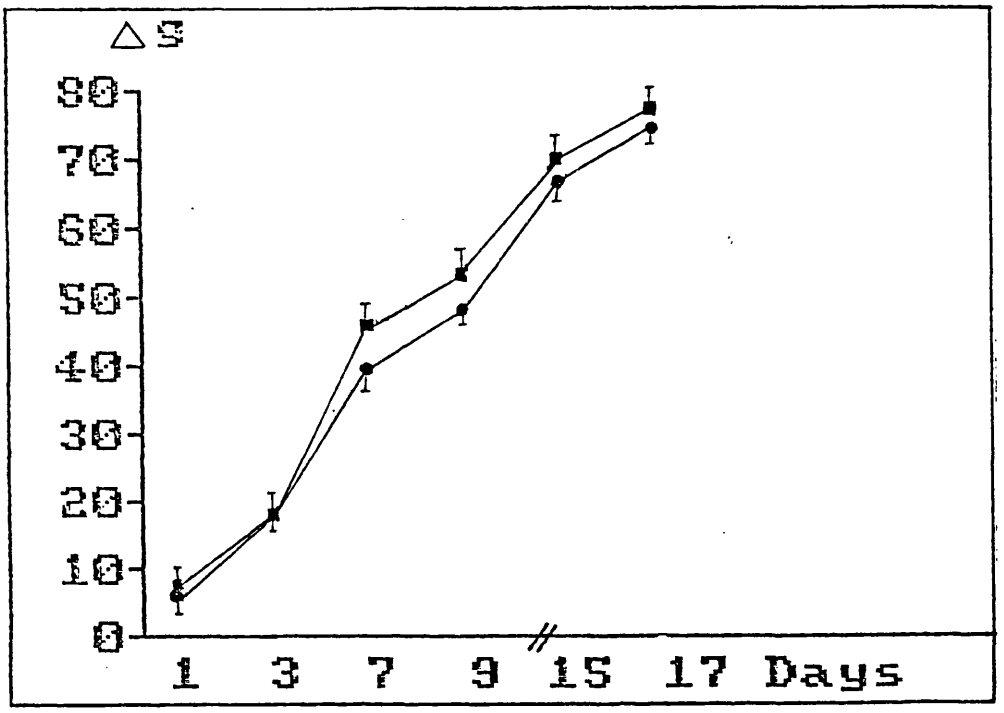


Fig. 3.1.7. Changes in body weight of control (●—●) rats and rats treated daily with 200 mg/kg D-penicillamine p.o. (■—■) (n = 4 ± S.E.M.)

3.1.3 Kinetics of an Acute and Chronic Oral Dose of 200 mg/kg D-Penicillamine.

Initially, female, Sprague-Dawley rats weighing between 120-160g were given a single oral dose of D-penicillamine (200 mg/kg). Individual rats were bled, then sacrificed, at various time-points over the subsequent 72 hours (3 rats at each time-point). Serum was assayed for total D-penicillamine (ie. protein-bound, free and disulphides) by the method as described in Section 2.7.

Fig. 3.1.8 shows the serum concentration - time curve after a single oral dose of D-penicillamine (200 mg/kg). Peak serum concentrations of 72.9 $\pm$ 1.5 µg/ml (mean $\pm$ S.E.M.) were observed 45 minutes post-dosing. The decline in the serum concentration was biphasic with an initial half-life of 27.0 minutes followed by a much slower decline with a half-life of 51.02 hours. The serum concentrations in this phase at 24 hours were 13.1 $\pm$ 1.1 µg/ml.

The same procedure as above was repeated for rats that had been administered a daily, oral dose of D-penicillamine (200 mg/kg) for 15 days. The kinetics of the last dose of the treatment course are shown in Fig. 3.1.9 . Again, peak serum concentrations occurred

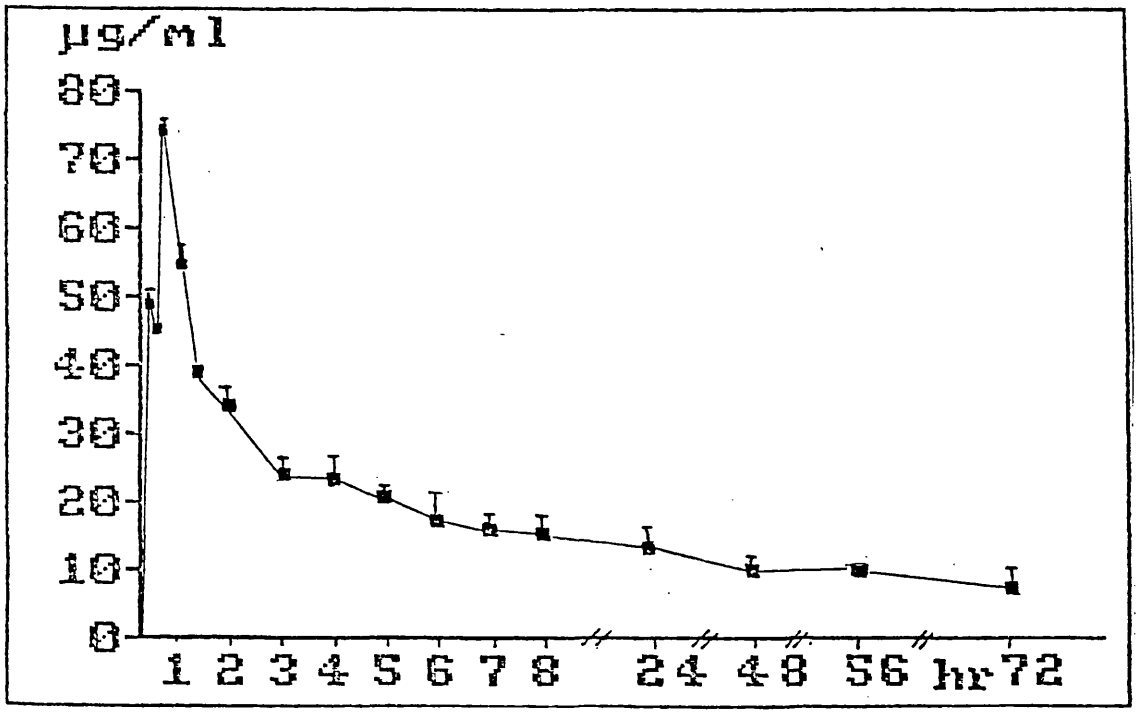


Fig. 3.1.8. Total D-Penicillamine in rat serum after a single, oral dose of D-penicillamine (200 mg/kg). (n = 3, mean ±S.E.M.)

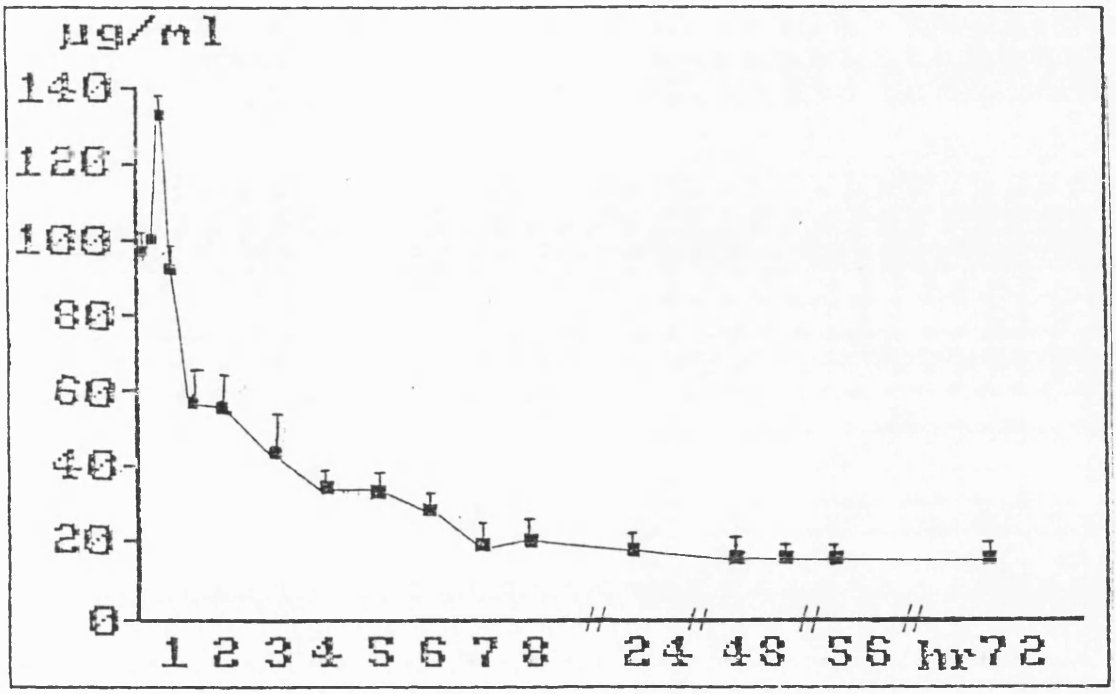


Fig. 3.1.9. Total D-penicillamine in rat serum after the last dose of D-penicillamine in a 15 day course of daily oral treatment with 200 mg/kg D-penicillamine.
(n = 3, mean ± S.E.M.)

at 45 minutes post-dosing, but the mean peak concentration was 130.6 ± 7.2 µg/ml. The initial and final phase half-lives were 51.5 minutes and 61.4 hours respectively.The serum concentrations in the second phase at 24 hours were 18.4 ± 1.5 µg/ml.

3.2 METABOLISM OF ^{14}C-D-PENICILLAMINE.

The availabilty of ^{14}C-D-penicillamine (kindly supplied by Lilly Research Centre) has allowed a more in-depth study into the plasma protein binding and metabolism of the drug. Except for a radiolabel study performed by Planas-Bohne (1981) using the racemic mixture ^{14}C-DL-penicillamine there are few other reports on the metabolism and disposition of the labelled drug. In addition, the identity of the major plasma metabolite(s) in rats remains speculative due to the lack of a selective, sensitive assay system. This section represents the results obtained from anesthetized animal studies and the findings in respect to metabolism in the rat.

3.2.1 Plasma Decay of ^{14}C-D-Penicillamine

Control and adjuvant arthritic rats (4 in each group) were intravenously dosed with ^{14}C-D-penicillamine (10 µCi; 200mg/kg). For methodology see Section 2.4.4. Radioactivity was measured in plasma and protein free ultrafiltrate over the subsequent 60 mins. Individual metabolites were identified by the radio-TLC system as described in Section 2.6.

Fig. 3.2.1 shows the decay of ^{14}C-D-penicillamine in plasma against time in control and adjuvant arthritic rats. Radioactive counts were significantly lower in the AA rats ($p<0.05$) during, but not after, the 4-5 minute equilibration period. The plasma radioactive decay was bi-exponential in both cases. The short rapid half-life was longer in the AA rats (4.52 min.) than in the controls (3.14 min.). The second phase half-lives, however, showed no significant difference between controls and AA rats (2.13 hours and 2.33 hours respectively - see table 6). In order to obtain truly accurate figures for the final half-lives, plasma decay should be measured over a time period twice the half-lfe value. However, this was not practically possible.

3.2.2 Bound and Free D-Penicillamine.

By measuring the counts in plasma and protein-free ultrafiltrate within 60 mins. post-dosing, the percentage of the drug bound to plasma proteins can be calculated. The initial plasma protein binding of D-penicillamine in the AA rat was 43.3 $\pm$ 1.9%, significantly lower ($p<0.05$) than in the controls where plasma protein was found to be 62.5 $\pm$ 5.8%. However, both groups rapidly reached an equilibrium of around 55% protein binding at 1 hour post-dosing. (Fig. 3.2.2). Although it was not feasable to assay for total

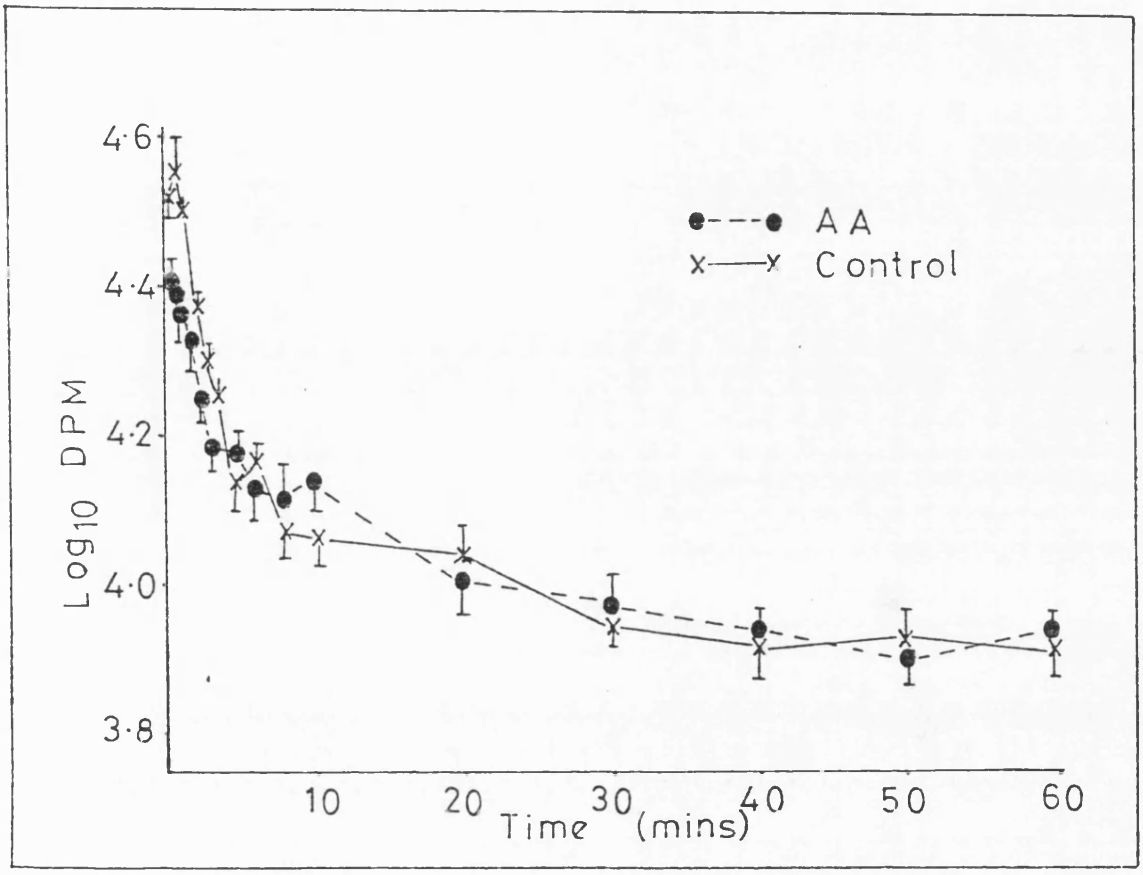


Fig. 3.2.1. Plasma radioactive decay after an intravenous dose of ^{14}C-D-penicillamine (200 mg/kg:10 µCi) in normal and adjuvant arthritic rats (n=4, mean ± SEM).

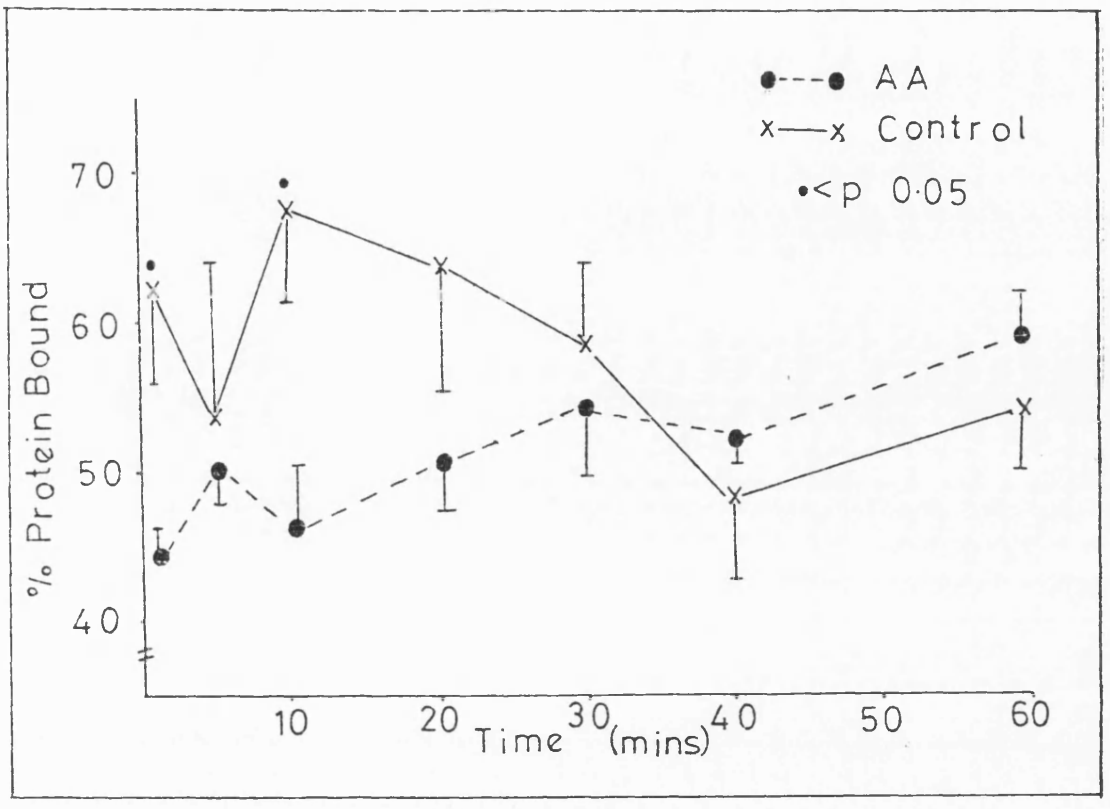


Fig. 3.2.2. Binding of ^{14}C-D-penicillamine to plasma proteins in normal and adjuvant arthritic rats (n=4, mean ± SEM).

	Control	AA
t1/2 (initial)	3.14 min	4.52 min
t1/2 (final)	2.13 hours	2.33 hours
^{14}C protein binding (60 sec)	62.5±5.8%	43.4±1.9%
^{14}C protein binding (30 min)	56.9±3.7%	54.5±3.1%

Table 6 Disposition of ^{14}C D-Penicillamine in Control and Adjuvant Arthritic Rats (n=4)
(n=4, mean ± S.E.M.)

D-penicillamine in these samples (sample volume = 0.3 ml whole blood), it can be reasonably assumed that the concentration will be similar to that observed after an acute intravenous dose of "cold" D-penicillamine (Section 3.3, Fig. 3.3.1). Thus a percentage binding of 55% relates to a bound plasma concentration of between 40-50 µg/ml at 1 hour.

In addition to the radiolabel binding study, free penicillamine was measured by HPLC in the serum of normal rats intravenously dosed with 200 mg/kg D-penicillamine. Table 7 shows that the free thiol gradually disappears from the blood until at 3 hours post dosing it is undetectable due to the limit of sensitivity of the HPLC system which was 0.1 µg/ml.

3.2.3. Metabolite Identification.

The results in Fig. 3.2.3 show that in both the AA and controls the major plasma metabolite was penicillamine disulphide with a small proportion of free penicillamine present initially. However, penicillamine-cysteine disulphide was undetectable in the plasma of either group. It should be remembered that although the method of identification may be qualitative, it is certainly not accurate or sensitive enough to be quantitative. Hence penicillamine cysteine may well be present in some amount but not sufficent

Time (hrs) (post-dose)	Conc. free PSH (µg/ml)	Conc. total PSH (µg/ml)	%free
0.25	11.26±0.1	272.0±1.5	4.1
0.5	5.87±0.1	127.4±11.6	4.6
1.0	2.84±0.05	80.7±13.2	3.5
2.0	0.8 ± 0.05	26.4±4.4	3.0
3.0	not detected	------	---

Table 7. Percentage of free D-penicillamine after a 200 mg/kg intravenous dose (n=3, mean ± S.E.M.)

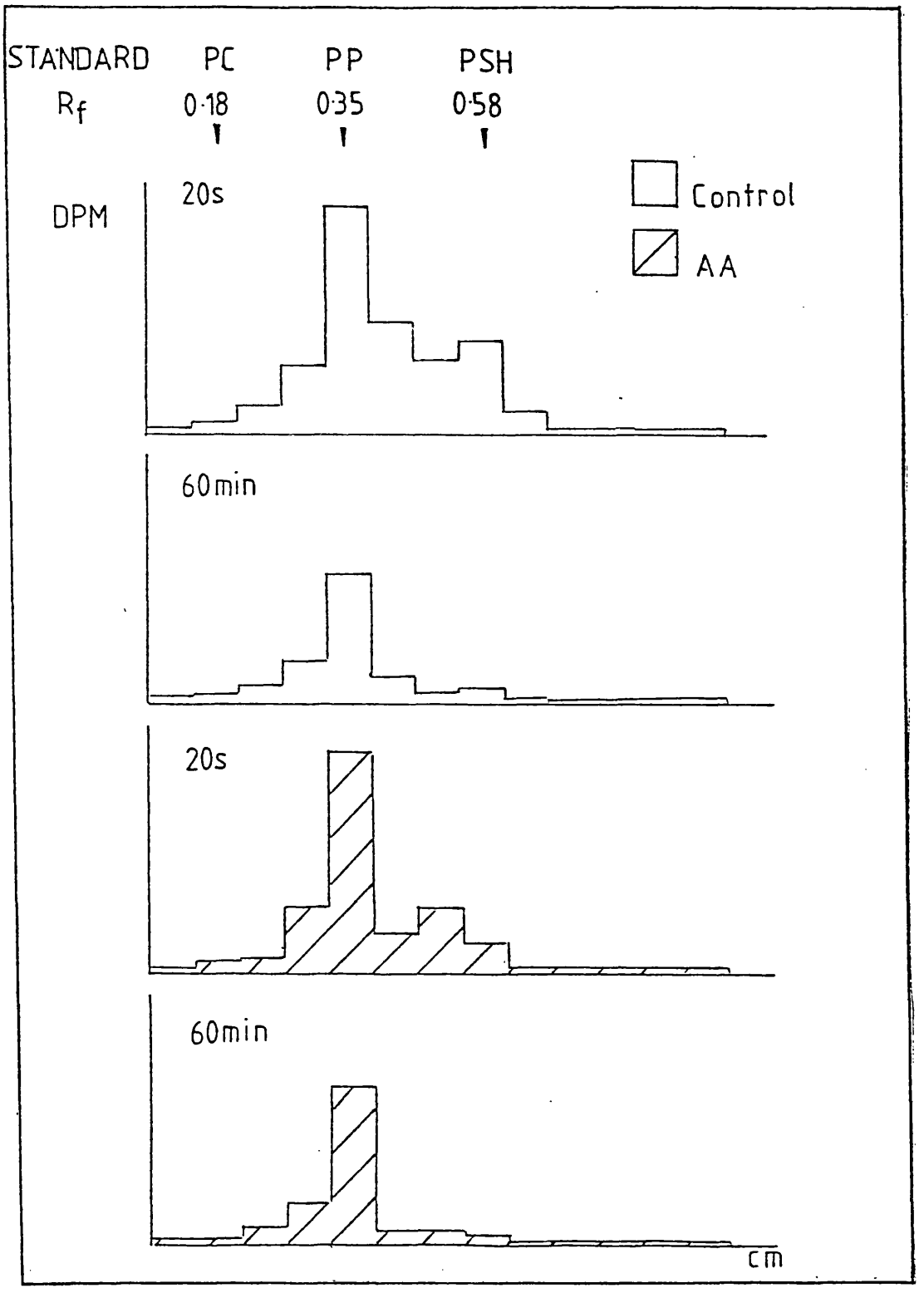


Fig. 3.2.3. Radio-TLC of ultrafiltrate obtained from plasma from normal and adjuvant arthritic rats at (i) 20 sec. and (ii) 60 mins after an intravenous dose of ^{14}C-D-penicillamine

quantities for detection. However, if the presence of penicillamine cysteine is considered to be just indistinguishable from backgound counts, an estimate of the minimum PSSP:PSSC ratio can be obtained from DPM at the PSSP spot divided by 2 (to account for twice the penicillamine content) related to the background DPM. This gives a minimum estimated PSSP:PSSC ratio of 14:1 in the rat.

3.3 THE PHARMACOKINETICS OF D-PENICILLAMINE AND ITS DISULPHIDES.

3.3.1 Total Serum D-Penicillamine Levels

The study of the pharmacokinetics and metabolism of D-penicillamine has in the past been limited by the absence of a reliable analytical technique for separation and quantification of the metabolites of the drug (See section 1.1.5). In order to overcome this problem, the metabolism and disposition of D-penicillamine, penicillamine disulphide (the major metabolite identified in rat serum in Section 3.2) and penicillamine cysteine (the principle metabolite in humans), was investigated by the measurement of total serum D-penicillamine levels after oral and intravenous dosing with the individual compounds. For comparitive purposes the disulphides were administered at equimolar equivalents of 200mg/kg D-penicillamine, ie 202 mg/kg penicillamine disulphide, and 362 mg/kg penicillamine cysteine.

Female Sprague-Dawley rats weighing between 120-160g were given either a single intravenous or oral dose of one of the D-penicillamine compounds at the dosage levels stated above. Blood was then taken and the rat

sacrificed at various time points over the subsequent 72 hours (3 rats per time point). Serum was assayed for total D-penicillamine (Section 2.7).

The pharmacokinetic profiles of D-penicillamine after oral and intravenous dosing are shown in Fig. 3.3.1. Peak serum concentration after oral dosing occurred at 45 mins post-dosing and was 72.9 $\pm$ 1.5 µg/ml. The decline in the serum drug concentration was biphasic with an initial rapid half-life of 27.0 mins followed by a much slower decline of 51.0 hours. The areas under the curve (AUC) value (0-8 hours), as calculated using the trapezoidal rule, after oral and intravenous dosing were 214 $\pm$ 17.1 and 331 $\pm$ 18.3 mg.h/L respectively, giving an oral biovailabilty of 64.8%.

Fig. 3.3.2 shows the fate of penicillamine disulphide (PSSP) after oral and intravenous dosing and the kinetic profiles are shown in comparison with D-penicillamine in Figs. 3.3.3 and 3.3.4. Penicillamine disulphide is poorly absorbed from the gut, peak serum concentrations being only 4.67 $\pm$ 0.7 µg/ml and occurring 1 hour after administration as compared to 72.9 $\pm$ 1.5 µg/ml at 0.75 hours for D-penicillamine ($p<0.001$) The oral data shows a monophasic decline in serum penicillamine levels, in contrast to the biphasic decline exhibited after intravenous dosing. The values for initial and terminal phase half-lives calculated

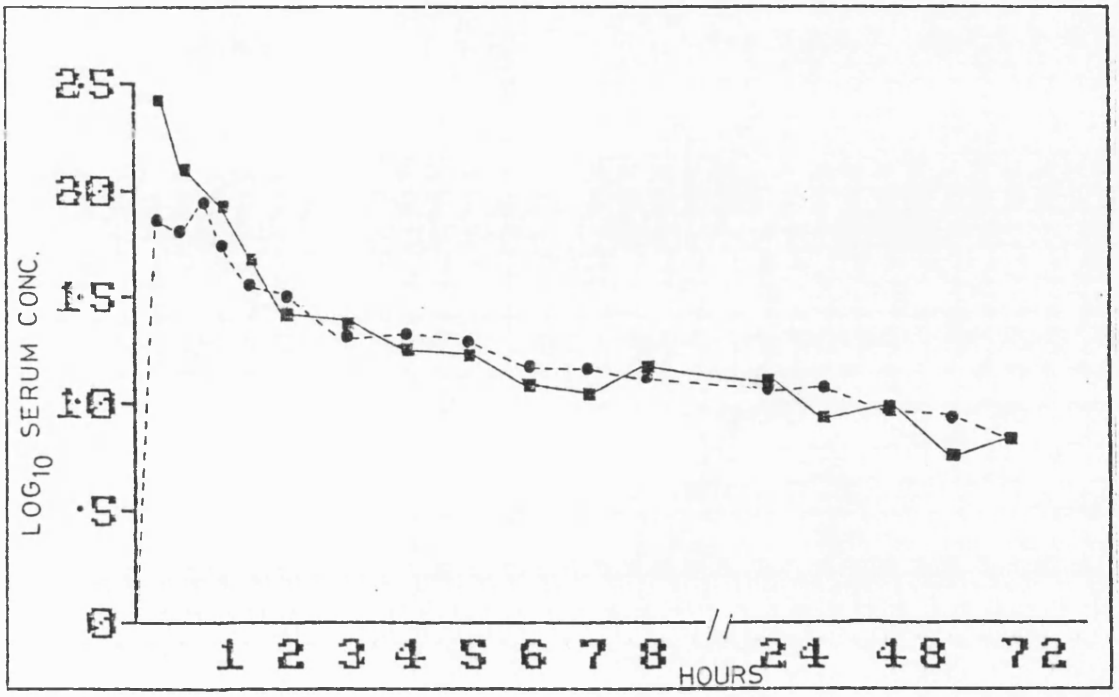


Fig. 3.3.1 Log_{10} total D-penicillamine in serum vs. time after a single oral (●- -●) and i.v. (■—■) dose of 200 mg/kg PSH. Values are the mean of 3 rats. For simplification of the graph, SEM are not shown, but are indicated in Appendix 1

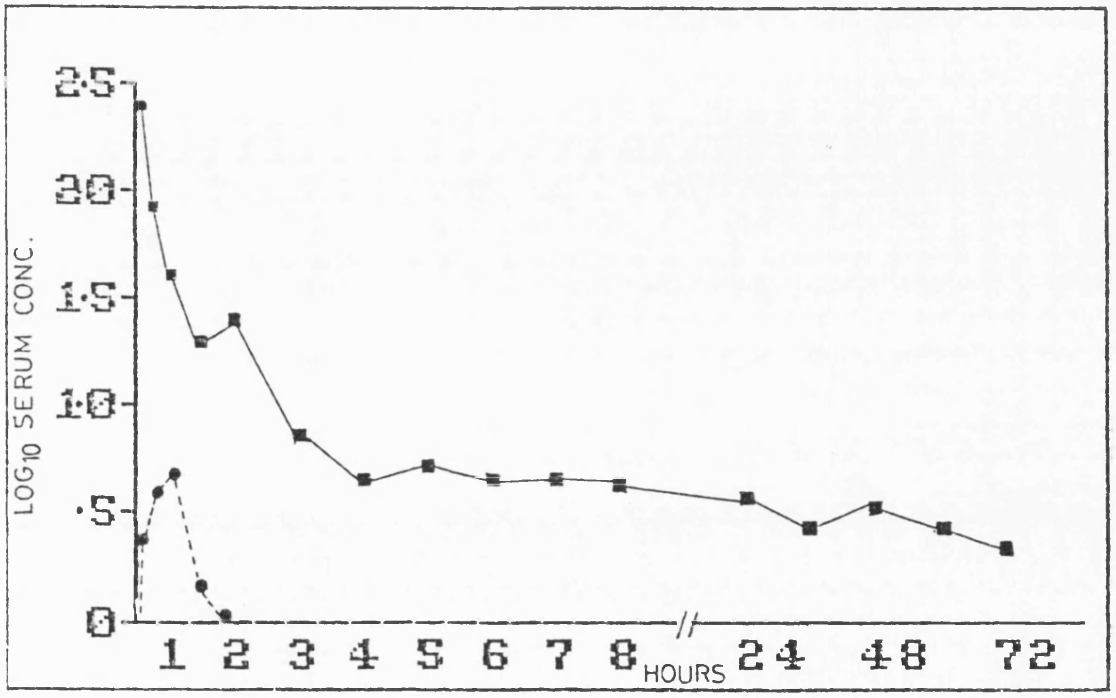


Fig. 3.3.2 Log_{10} total D-penicillamine in serum vs. time after a single oral (●--●) and i.v. (■—■) dose of 202 mg/kg PSSP. Values are the mean of 3 rats. For simplification of the graph, SEM are not shown, but are indicated in Appendix 2

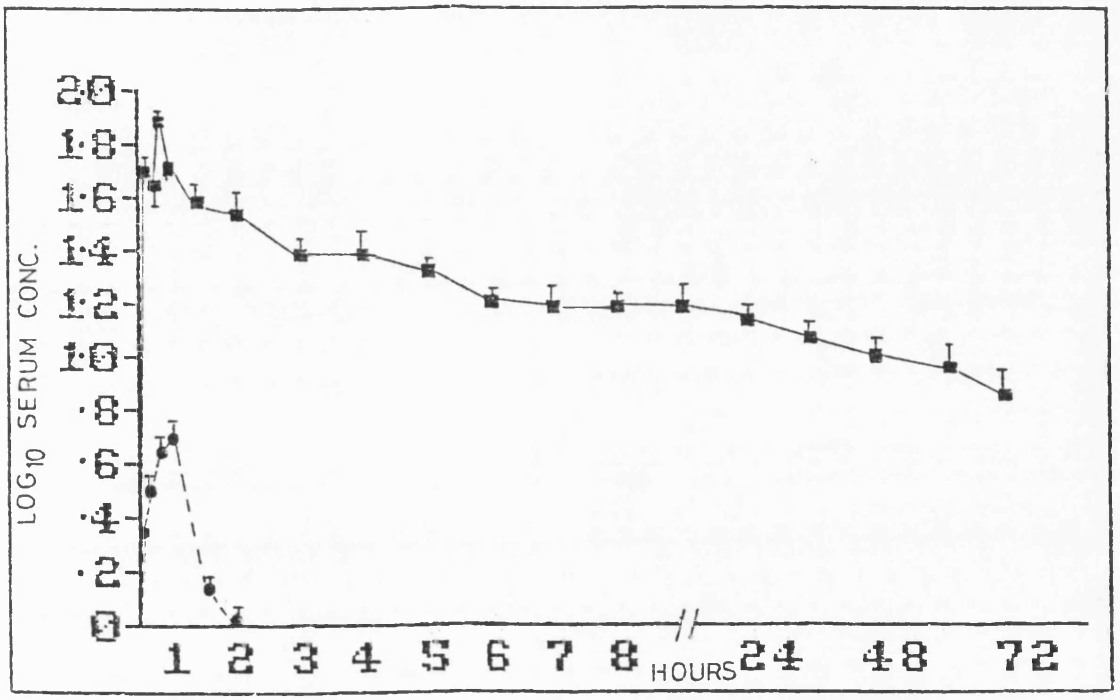


Fig. 3.3.3 Log_{10} total D-penicillamine in serum vs. time after a single oral dose of 200 mg/kg PSH (■——■) and 202 mg/kg PSSP (●--●). Values are the mean of 3 rats ± SEM.

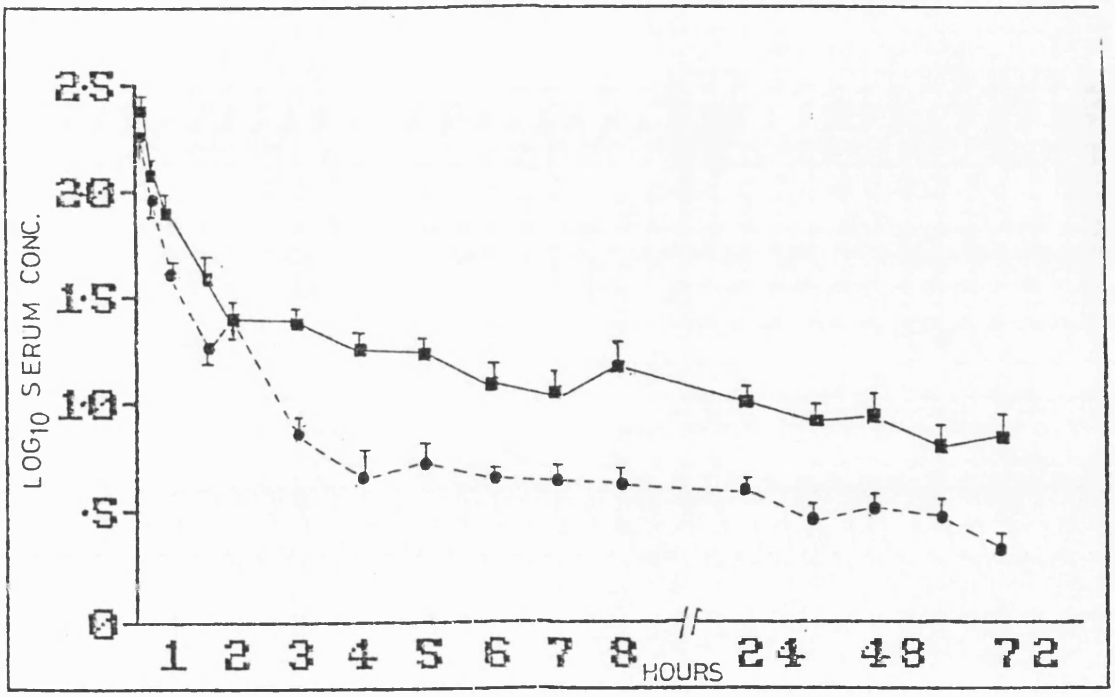


Fig. 3.3.4 Log_{10} total D-penicillamine in serum vs. time after a single i.v. dose of 200 mg/kg PSH (■—■) and 202 mg/kg PSSP (●--●). Values are the mean of 3 rats ± SEM.

from the iv results are 18.1 mins and 62.7 hours respectively. The AUC after oral dosing was only 5.2 ± 0.8 mg.h/L and 214.9 ± 12.3 mg.h/L after i.v., both values considerably lower than those obtained after D-penicillamine administration (214 ±17.1 and 331 ±18.3 respectively).

Figs. 3.3.5, shows the kinetic profiles obtained after acute oral and intravenous penicillamine cysteine (PSSC) treatment. Figs. 3.3.6 and 3.3.7 show these kinetics in comparison to the D-penicillamine data. Peak serum D-penicillamine concentrations occur at 1 hour post oral dosing with PSSC and are significantly lower at 55.7 ± 5.7 µg/ml as compared to 72.9 ± 1.5 µg/ml at 0.75 hour for D-penicillamine ($p<0.05$). This reduced absorption of the drug is again highlighted by the AUC for penicillamine cysteine being only 184.9 ± 13.3 mg.h/L after oral administration as compared to 214 ± 17.1 for D-penicilamine. However, the AUC_{iv} of 385 ± 25.2 mg.h/L, is not significantly different from the AUC of 331 ± 18.3 obtained after iv treatment with D-penicillamine. The biphasic decline in total serum D-penicillamine is apparent, with an initial half-life of 24.9 mins and a terminal phase half-life of 35.8 hours.

Free D-penicillamine was detectable only in samples from animals treated with the parent compound, and the

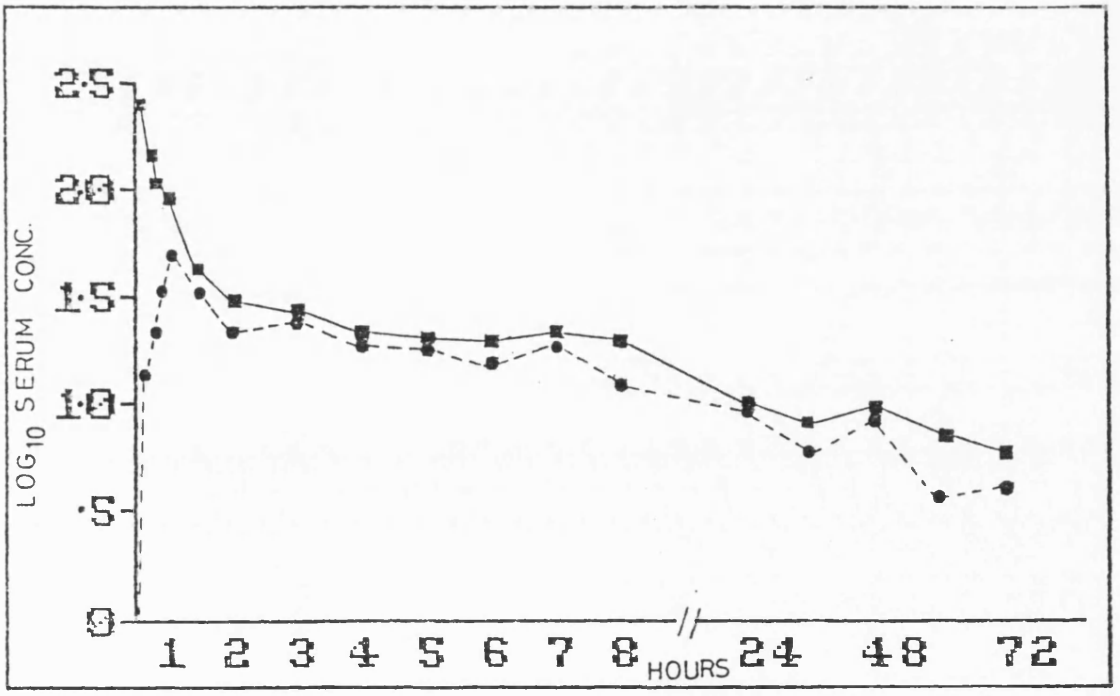


Fig. 3.3.5 Log_{10} total D-penicillamine in serum vs. time after a single oral (●--●) and i.v. (■—■) dose of 362 mg/kg PSSC. Values are the mean of 3 rats. For simplification of the graph, SEM are not shown, but are indicated in Appendix 3

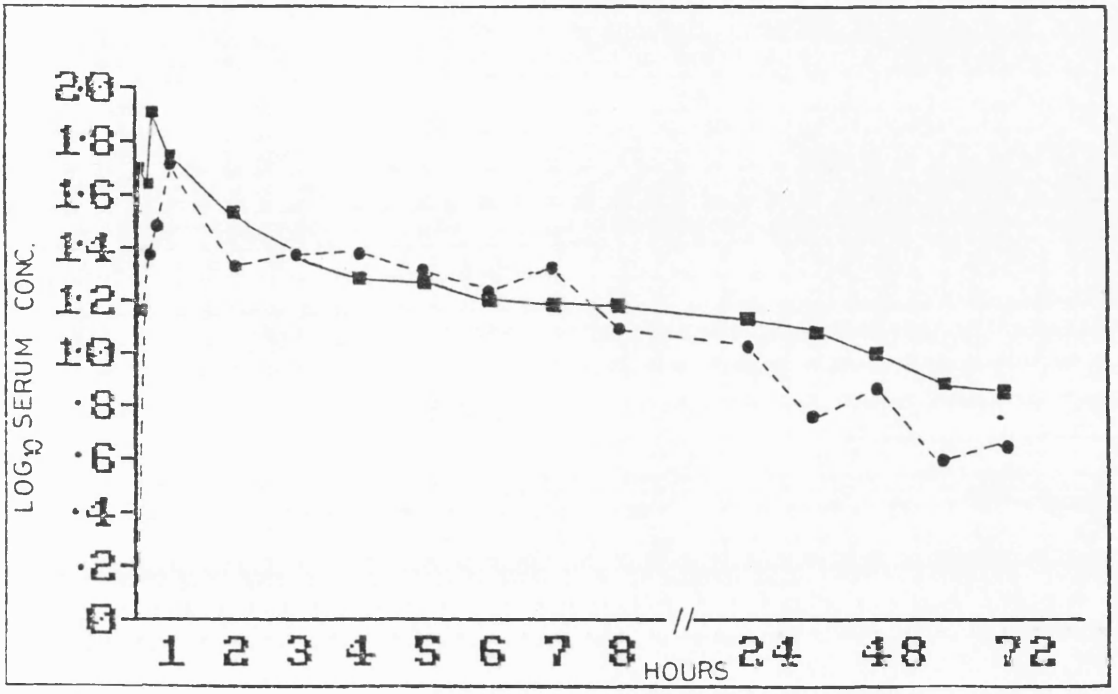


Fig. 3.3.6 Log_{10} total D-penicillamine in serum vs. time after a single oral dose of 200 mg/kg PSH (■——■) and 362 mg/kg PSSC (●--●). Values are the mean of 3 rats. For simplification of the graph, SEM are not shown but are indicated in Appendix 1 and 3

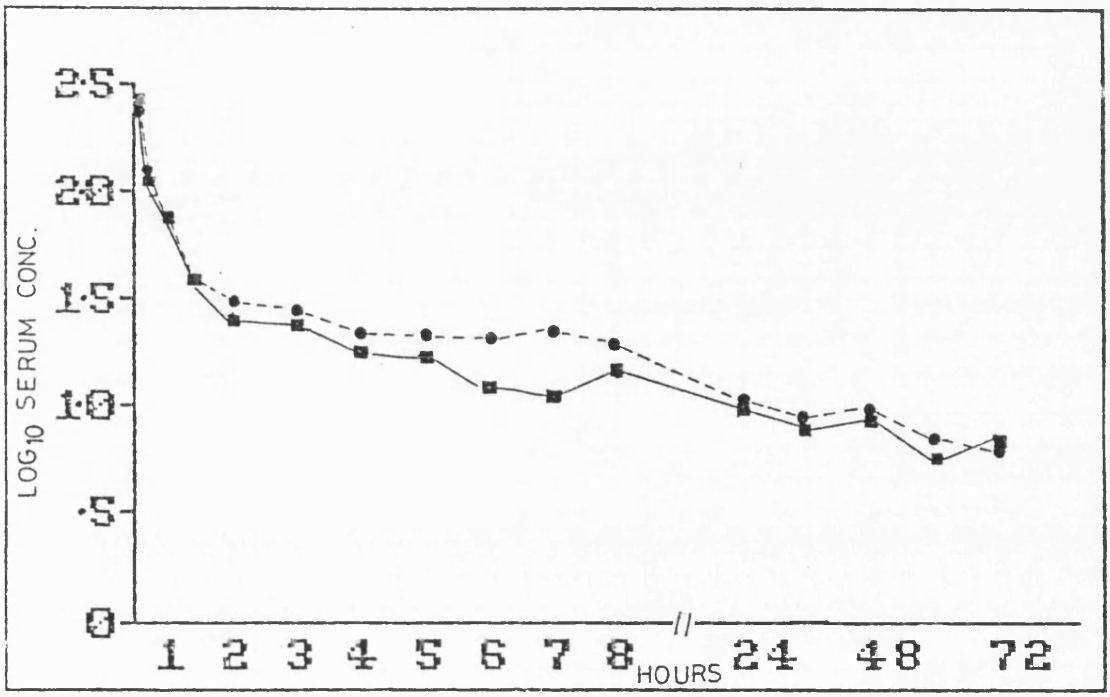


Fig. 3.3.7 Log_{10} total D-penicillamine in serum vs. time after a single i.v. dose of 200 mg/kg PSH (■—■) and 362 mg/kg PSSC (●--●). Values are the mean of 3 rats. For simplification of the graph, SEM are not shown but are indicated in Appendix 1 and 3

rate of disappearance of the free drug is shown in Table 7, Section 3.2.

A summary of the pharmacokinetic parameters obtained after a single oral and iv dose of D-penicillamine and its disulphides is shown in Table 8.

3.3.2. Urinary Excretion of D-Penicillamine.

Female Sprague-Dawley rats weighing between 120-160g were intravenously dosed with either D-penicillamine (200 mg/kg), penicillamine disulphide (202 mg/kg) or penicillamine cysteine (362 mg/kg). Four rats per dosage group were used. Urine was collected over the subsequent 24 hours and assayed for D-penicillamine after reduction to the free thiol as described in Section 2.7.

The excretion of D-penicillamine in the urine after intravenous administration of penicillamine disulphide and penicillamine cysteine, as compared with D-penicillamine are shown in Figs. 3.3.8 and 3.3.9 respectively. With all three compounds, the majority of the excretable D-penicillamine was recovered in the urine 0-4 hours post-dosing. The urinary excretion profile after penicillamine cysteine treatment is not significantly different from that after D-penicillamine itself, total excretion in 48 hours being 41.2 $\pm$ 2.5%

	PSH	PSSP	PSSC
AUC_{8h} oral (mg.h/L)	214±17.1	5.2±0.8	185±13.3
Peak serum conc. (oral) (mg/L)	72.9±1.5	4.67±0.7	55.7±5.7
AUC_{8h} i.v. (mg.h/L)	331±18.3	215±12.3	385±25.2
Peak serum conc. (i.v)(mg/L)	329	351	350
Initial phase half-life (mins)	27.0	18.1	24.9
Terminal phase half-life (hrs)	51.0	62.7	35.8
Urinary Excretion (% Dose/48 hr)	41.2±2.5	69.1±5.0	47.7±4.9

Table 8. Pharmacokinetic Parameters following a Single Oral and Intravenous Dose of D-Penicillamine (PSH), Penicillamine Disulphide (PSSP) and Penicillamine Cysteine (PSSC). All doses were the equivalent of 200 mg/kg PSH. (mean ± SEM).

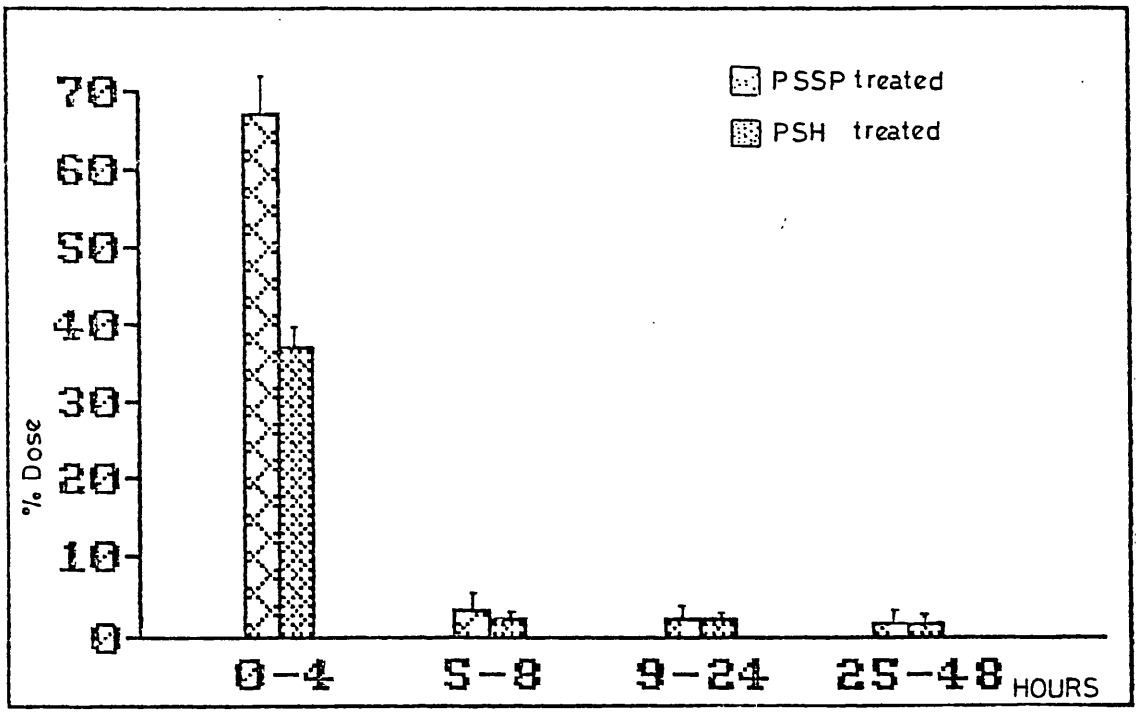


Fig. 3.3.8 Urinary excretion of total D-penicillamine in the 48 hour period after an intravenous dose of 200 mg/kg PSH and 202 mg/kg PSSP. (n=4, mean ± SEM)

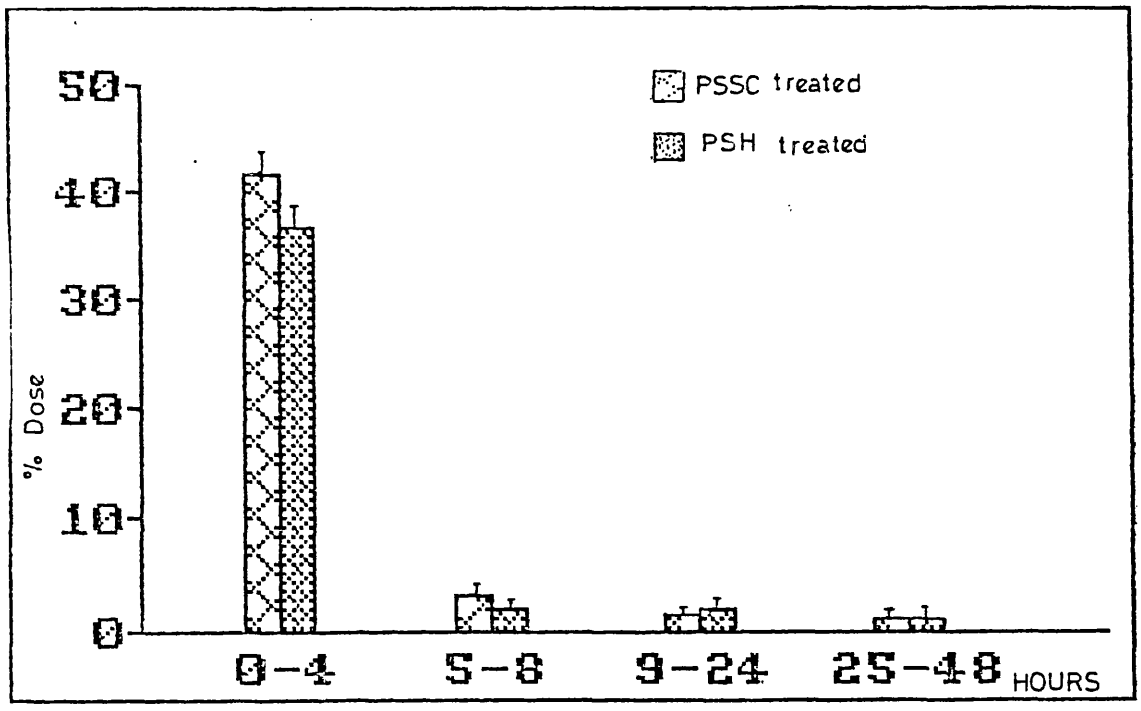


Fig. 3.3.9 Urinary excretion of total D-penicillamine in the 48 hour period after an intravenous dose of 200 mg/kg PSH and 362 mg/kg PSSC. (n=4, mean ± SEM)

and 47.7 $\pm$ 4.9% respectively. However, total urinary output after penicillamine disulphide was significantly higher than after D-penicillamine in the 0-4 hour period post-dosing ($p<0.02$). Total excretion over the 48 hour time-course was 69.1 $\pm$ 5.0% of the dose given (Table 8).

3.4. THE EFFECTS OF D-PENICILLAMINE AND PENICILLAMINE CYSTEINE ON ADJUVANT ARTHRITIS.

As reviewed in Section 1.3, there are very few animal models of arthritis in which D-penicillamine exhibits disease modifying activity. This may be, in part, due to differences in metabolite formation in humans and the rat. Section 3.2 has shown that the major metabolite in the blood of the rat is penicillamine disulphide with small amounts of free parent drug. No penicillamine cysteine was detectable, the predominantly occurring metabolite occurring in humans. It has been suggested (Nakaike et al, 1983), that penicillamine-cysteine may have an immunomodulatory effect and therefore may be the active species in RA therapy with D-penicillamine. Hence it was of interest to assess the effects of this compound on the development of adjuvant arthritis, the most popular model for assessing potential anti-rheumatic agents and one in which D-penicillamine itsef appears to have little disease modifying activity.

3.4.1 Effects on Arthritic Parameters

Arthritis was induced in female Sprague-Dawley rats by an injection of 0.05 ml Complete Freund's Adjuvant containing heat killed M. tuberculosis (2.5 mg/ml) into the right hind paw. The development of the disease was

monitored by increase in foot volume, changes in body weight and arthritic score over an 18 day period. The effects of daily, oral treatment of D-penicillamine (200 mg/kg) and penicillamine cysteine (362 mg/kg) commencing 1 day prior to AA induction, on each of these parameters was obseved. Control AA rats were dosed with vehicle only (n = 6 in each treatment group).

An intense but transient inflammatory response occurred immediately after injection of the adjuvant. This initial phase of acute inflammatory response was local and produced no other obvious reactions in the animals other than a marked increase in foot volume (Fig. 3.4.1). Over the subsequent 7 days after innoculation, the injection site gradually necrosed but the animals retained a generally healthy appearance, with no further increase in foot volume. However, 9 days into the course, a marked change began to occur. The injected hind paw began to swell and extensive arthritic inflammation appeared in areas remote from the injection site. The distal small joints of both hind paws were affected with varying degrees of severity and mobility. At this time, swelling of the left hind paw also became apparent. The forepaws and tails of the rats were commonly distended and in the most drammatically responding animals, the eyes, nose and ears became deformed. In all animals that developed

the disease, a retardation in weight gain was recorded (Fig. 3.4.3) and, on occassions, actual weight loss.

As illustrated in Figs. 3.4.1 and 3.4.2 treatment with either D-penicillamine or penicillamine cysteine failed to significantly modify the swelling in right or left hind paw. Also, there was no effect of either compound on the retardation of weight gain, AA animals increasing in body weight by only 44g as compared to 77g in normal rats (Figs. 3.4.3a and b). Arthritic scores in both AA controls and the two drug treated groups are shown in Fig. 3.4.4. AA control rats reached a maximum score of 9.1 $\pm$ 0.4 on day 18. Daily oral treatment with either D-penicillamine or penicillamine cysteine did not significantly alter the score, being 8.3 $\pm$ 0.4 and 8.1 $\pm$ 0.6 respectively ($p>0.05$) at the end of the course.

3.4.2 Total Serum SH Levels During the Development of Adjuvant Arthritis

Induction of arthritis and daily oral treatment with D-penicillamine (200 mg/kg) and penicillamine cysteine (362 mg/kg) was as described above. Rats were bled and sacrificed on days 1, 3, 5, 7, 9, 15, 17 after induction of arthritis (4 rats per treatment group per day). Serum was then assayed for total sulphydryl

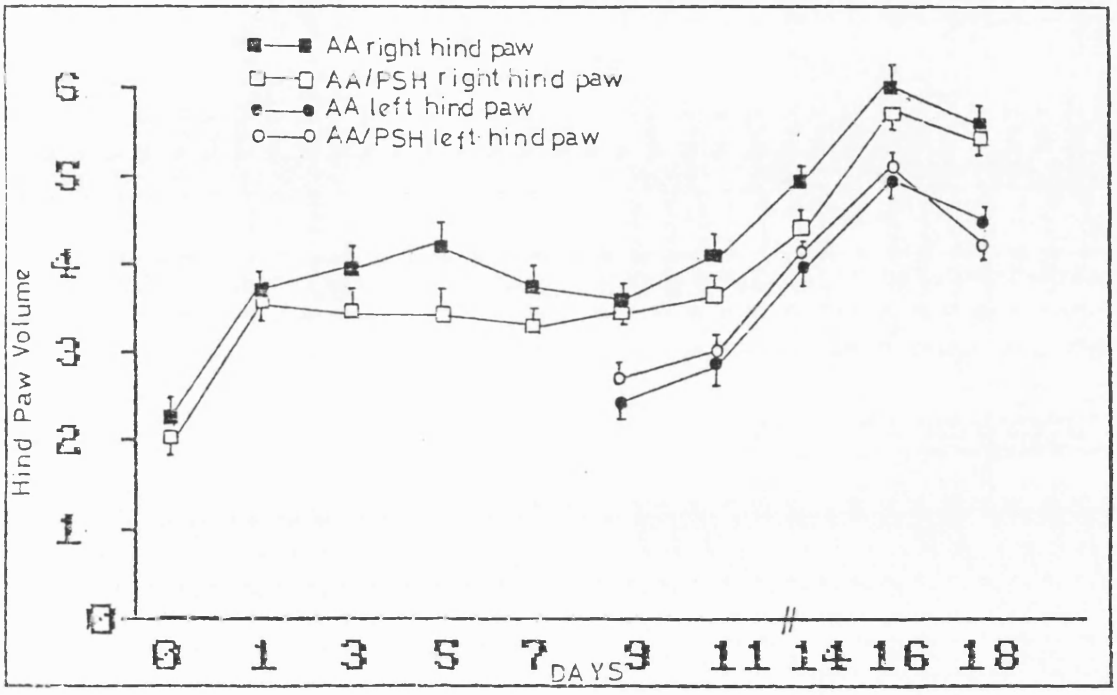


Fig. 3.4.1 The effect of D-penicillamine (200 mg/kg/day, p.o.) on the hind paw volume during the development of AA. (n=6, mean ± SEM).

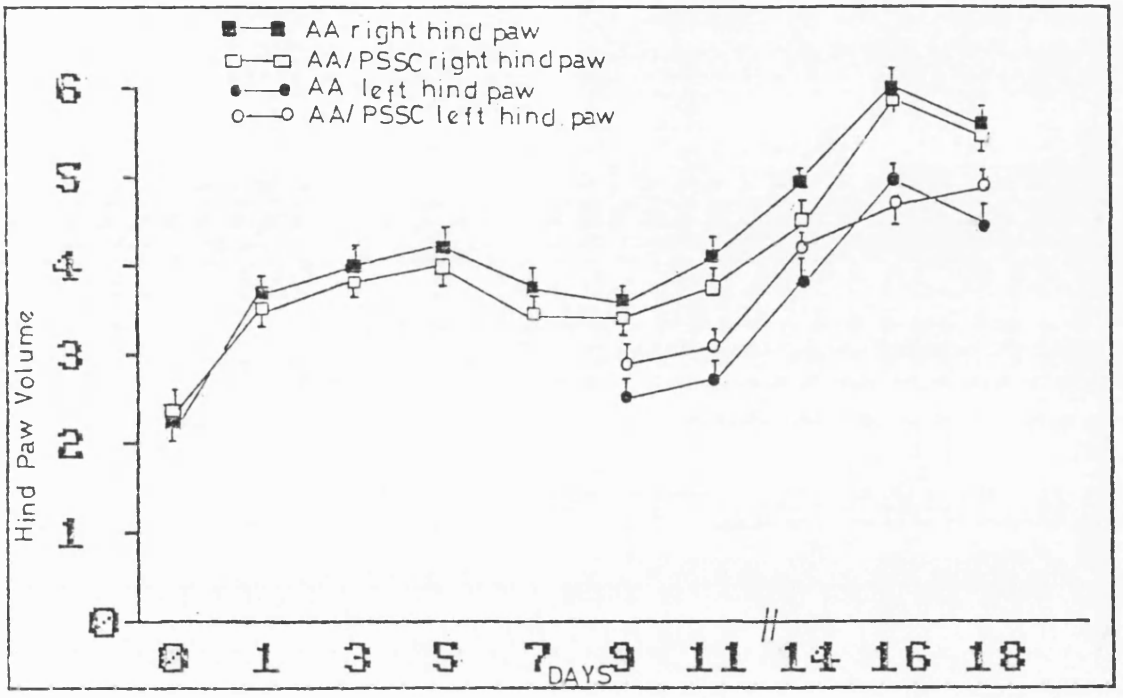


Fig. 3.4.2 The effect of penicillamine cysteine (362 mg/kg/day, p.o.) on the hind paw volume during the development of AA. (n=6, mean ± SEM).

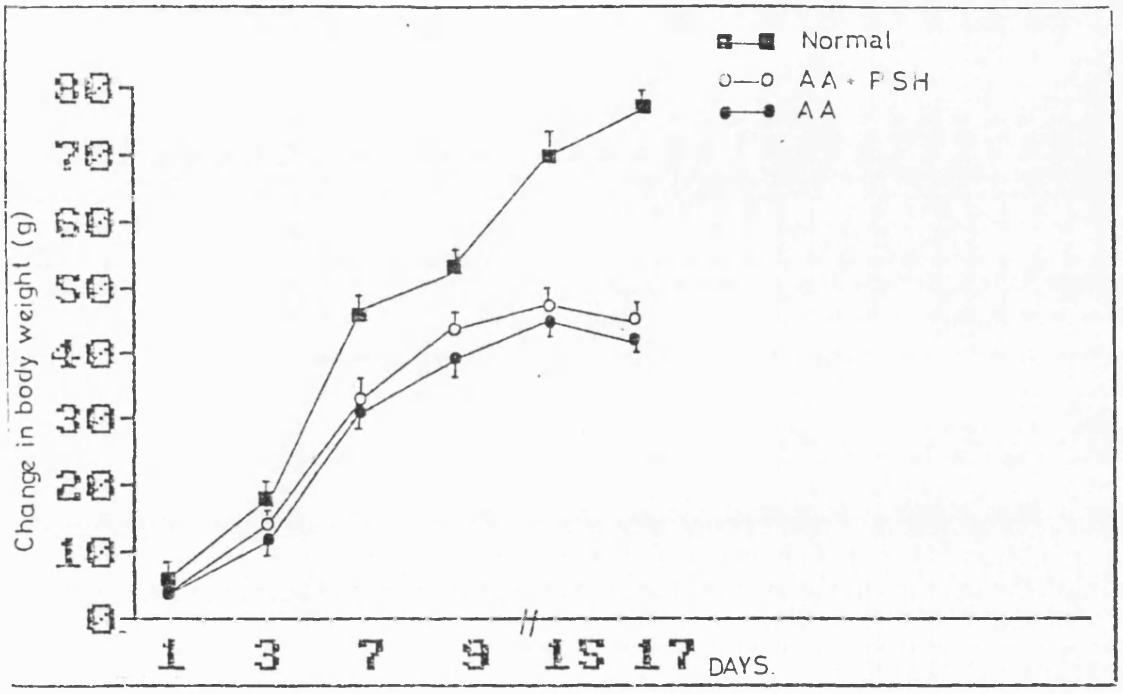


Fig. 3.4.3(a) The effect of D-penicillamine (200 mg/kg/day, p.o.) on the change in body weight during the development of AA. (n=6, mean ± SEM).

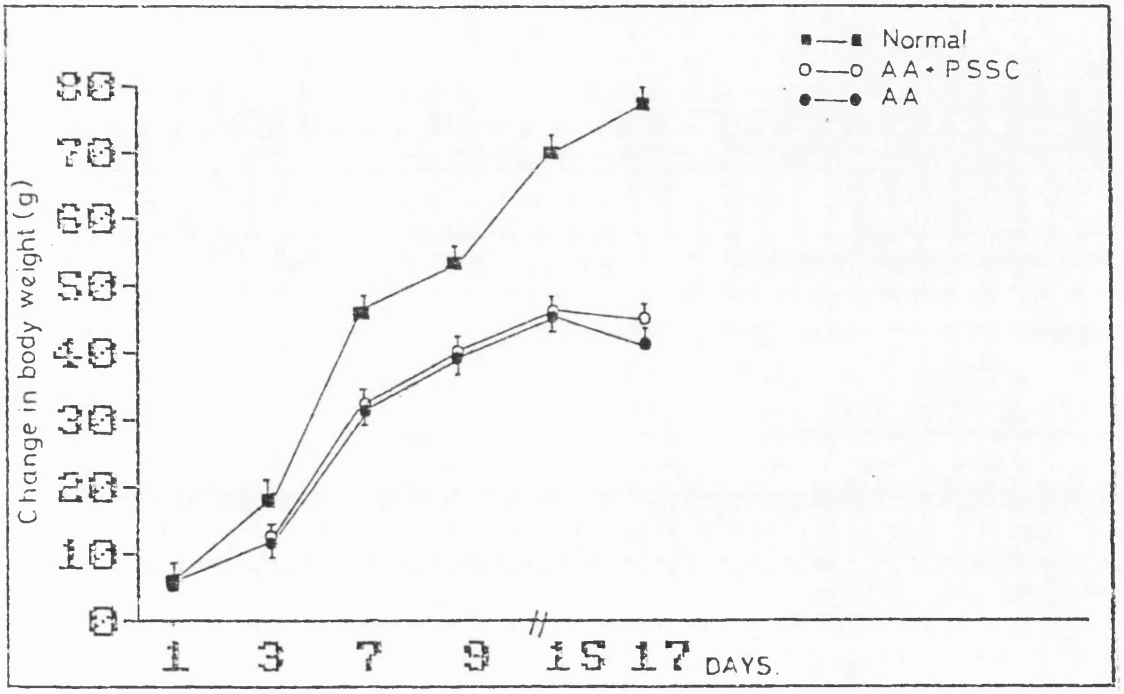


Fig. 3.4.3(b) The effect of penicillamine cysteine (362 mg/kg/day, p.o.) on the change in body weight during the development of AA. (n=6, mean ± SEM).

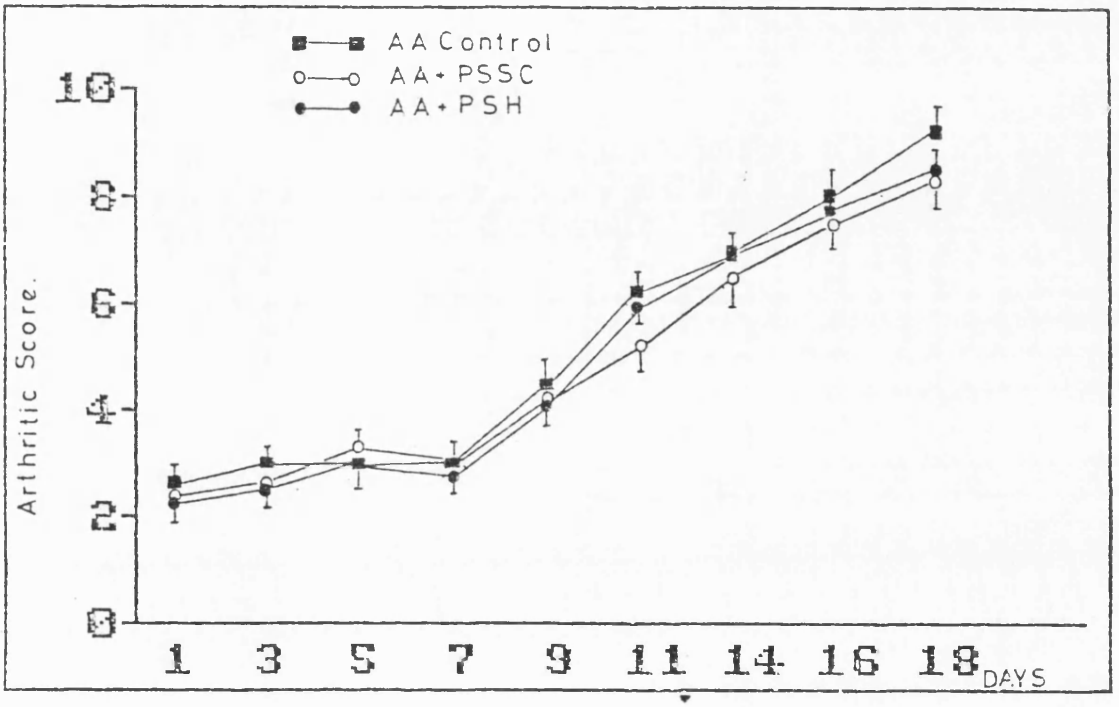


Fig. 3.4.4 The effect of D-penicillamine (200 mg/kg/day, p.o.) and penicillamine cysteine (362 mg/kg/day p.o) on the arthritic score during the development of AA. (n=6, mean ± SEM).

content as described in Section 2.5.

The serial changes in total serum sulphydryl levels in AA control rats and drug treated rats are shown in Fig. 3.4.5. In control AA rats, a rapid fall in serum SH levels occurred during the acute inflammatory phase ie from 53.9 $\pm$ 1.5 µg/ml on day 0 to 41.5 $\pm$ 0.6 µg/ml on day 3. Levels continued to fall to a minimum of 24.4 $\pm$ 0.6 µg/ml on Day 9, around the time of onset of AA. Thereafter, serum SH levels began to rise, but at the end of the 18 day course were only 33.0 $\pm$ 2.1 µg/ml, significantly lower than 0 time control ($p<0.01$)

Both D-penicillamine and penicillamine cysteine appeared to exacerbate the initial fall in serum sulphydryl levels in the acute inflammatory phase. On day 3 seum SH levels were significantly lower than the AA control rats; 37.5 $\pm$ 0.9 µg/ml after D-penicillamine treatment ($p<0.05$) and 29.9 $\pm$ 2.6 µg/ml ($p<0.02$) after treatment with penicillamine cysteine. At around the time of onset of AA (Day 9), serum SH levels after drug treatment were not significantly different from AA controls, but both PSH and PSSC treatment increased the the serum SH levels in AA rats towards normality, reaching values of 47.8 $\pm$ 1.9 µg/ml ($p>0.05$ as compared to normals) and 43.4 $\pm$ 2.1 µg/ml ($p<0.05$ as compared to normals) respectively on day 18.

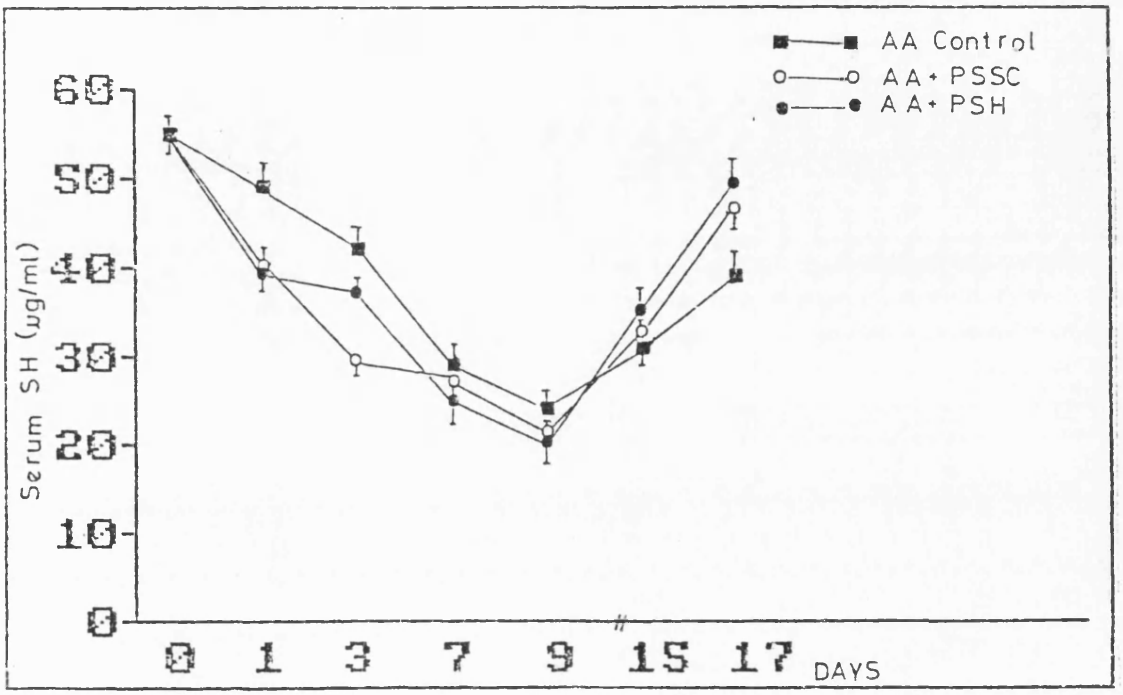


Fig. 3.4.5 The effect of D-penicillamine (200 mg/kg/day, p.o.) and penicillamine cysteine (362 mg/kg/day p.o) on total serum sulphydryl levels during the development of AA (n=4, mean ± SEM)

3.4.3 Serum and Urinary Copper Levels During the Development of AA

Induction of arthritis and daily oral treatment with D-penicillamine (200 mg/kg) and penicillamine cysteine (362 mg/kg) was as described above. Rats were placed in metabowls on days 1, 3, 5, 7, 9, 15 and 17 and urine collected over the subsequent 24 hours. (4 rats per treatment group per day). Animals were then bled and sacrificed at the end of the 24 hour period. Urine and serum were assayed for total copper content as described in Section 2.5.

The changes in serum copper levels during the development of AA are shown in Fig 3.4.6. An extremely rapid, drammatic rise in serum copper occurred between Days 0-1 increasing from a normal value of 1.1 $\pm$ 0.05 µg/ml to 2.4 $\pm$ 0.06µg/ml ($p<0.001$). Thereafter levels declined until on Day 7 they were 1.7 $\pm$ 0.2 µg/ml, just significantly different from normal ($p<0.05$). A second rapid increase in serum copper levels was apparent increasing to 2.6 $\pm$ 0.8 µg/ml on day 9 and these levels were maintained until the end of the course when serum copper was 2.76 $\pm$ 0.09 µg/ml on day 17 ($p<0.001$ AA vs normals). Both D-penicillamine and penicillamine cysteine returned the serum copper levels to normality in the acute inflammation phase ($p>0.05$ at day 7 for AA

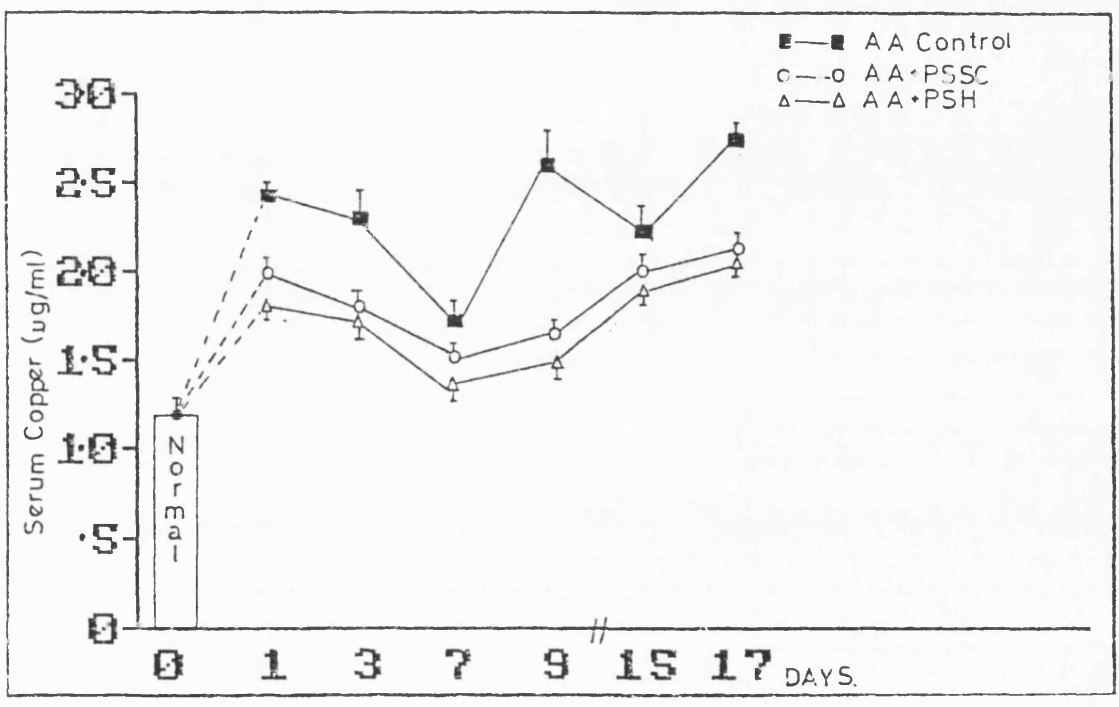


Fig. 3.4.6 The effect of D-penicillamine (200 mg/kg/day, p.o.) and penicillamine cysteine (362 mg/kg/day p.o) on serum copper levels during the development of AA (n=4, mean ± SEM)

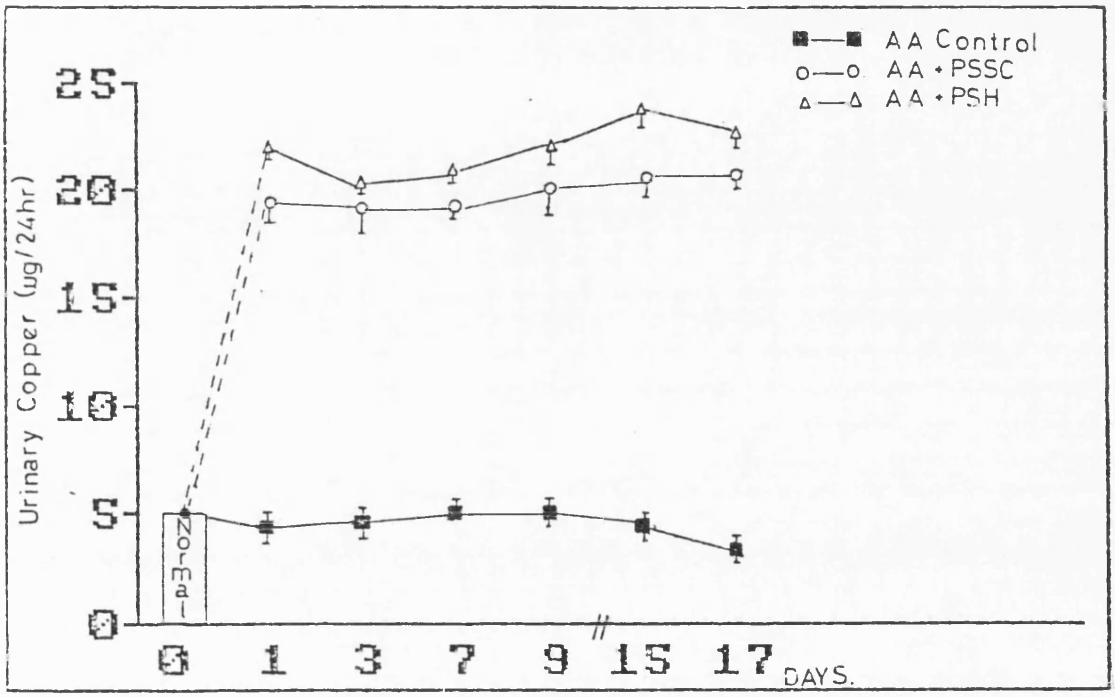


Fig. 3.4.7 The effect of D-penicillamine (200 mg/kg/day, p.o.) and penicillamine cysteine (362 mg/kg/day p.o) on urinary copper excretion during the development of AA (n=4, mean ± SEM)

rats treated with either compound vs normals). However, in the active phase of the disease D-penicillamine and penicillamine cysteine were unable to completely normalise serum copper levels values being 1.86 $\pm$ 0.06 µg/ml and 2.05 $\pm$ 0.01 µg/ml respectively ($p<0.01$ either compound vs normals) on day 17.

Fig 3.4.7 shows the urinary copper excretion profile throughout the adjuvant course. In the AA control rats, urinary copper excretion was not significantly different from values of 4.9 $\pm$ 0.9 µg/24 hr in normal rats ($p>0.05$). D-penicillamine and penicillamine cysteine caused a massive increase in urinary copper excretion rising to 22.9 $\pm$ 1.1 µg/24 hr and 18.2 $\pm$ 2.5 µg/24 hr respectively on day 1, as compared to 4.7 $\pm$ 0.98 µg/24 hr in AA control rats ($p<0.001$). These levels were maintained throughout the rest of the course.

3.4.4 Total Serum D-penicillamine Levels After Chronic Treatment of AA rats with PSH and PSSC.

Female Sprague-Dawley rats were orally dosed with D-penicillamine (200 mg/kg) or penicillamine cysteine (362 mg/kg) for 15 days. In AA rats, treatment commenced 1 day prior to innoculation. On day 15 of the course, rats were bled then sacrificed at various time

points over the subsequent 72 hours post-dosing (3 rats per time point). Serum was then assayed for total D-penicillamine as described in Section 2.7.

The kinetic profiles after daily oral dosing of each compound throughout the AA course are shown in Fig 3.4.8. Peak serum D-penicillamine levels were 121.4 $\pm$ 11.4 µg/ml at 45 mins after D-penicillamine treatment and 100.5 $\pm$ 6.7 µg/ml at 1 hour after dosing with penicillamine cysteine. The initial phase half-lives were not significantly different after treatment with D-penicillamine or penicillamine cysteine, being 51.4 mins and 58.3 mins respectively. However, the terminal phase half-life after penicillamine cysteine was 53.8 hours, considerably shorter than the half-life of 64.0 hours observed after D-penicillamine. Neither compound exhibited significant differences in pharmacokinetic parameters in adjuvant rats in comparison to normal rats (Table 9).

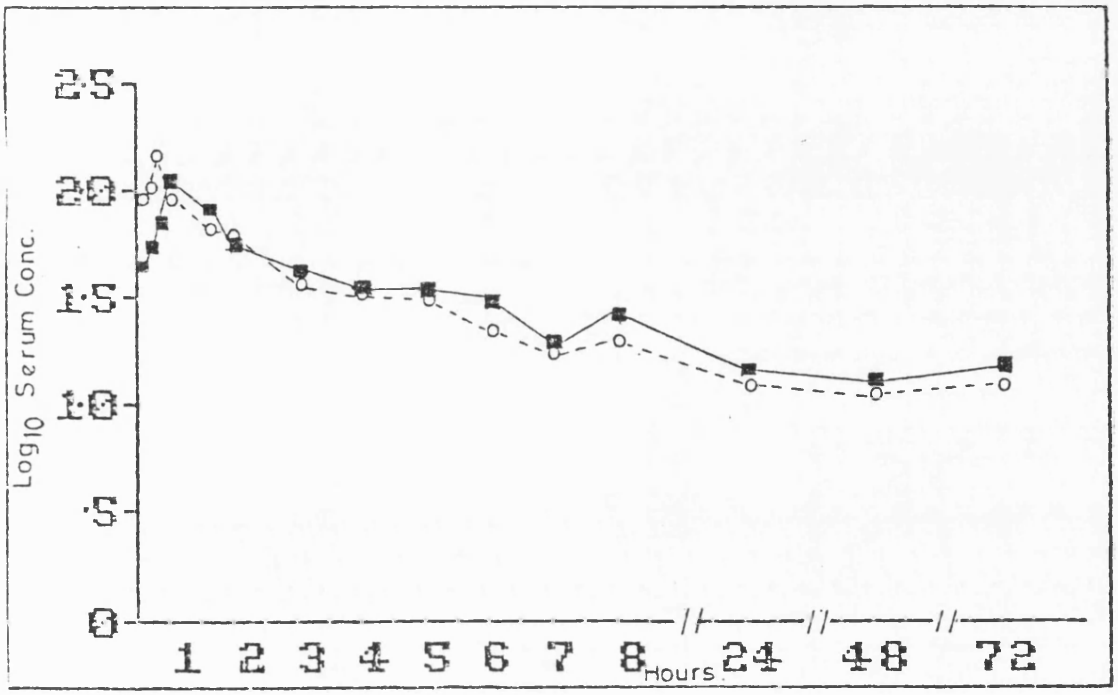


Fig. 3.4.8 Log_{10} total D-penicillamine in serum after the last oral dose in a 15 day treatment course of AA rats of 200 mg/kg PSH (●--●) or 362 mg/kg PSSC (■—■). Values are the mean of 3 rats. For simplification of the graph, SEM are not shown but are indicated in Appendix 4 and 5.

	PSH		PSSC	
	Normal	AA	Normal	AA
Peak Serum Conc. (µg/ml)	130.6 ±7.2	121.4 ±11.4	98.7 ±8.6	100.5 +6.7
AUC_{0-8} (mg.h/L)	359.4 ±42.8	340.5 ±28.9	312.6 ±27.7	318.9 ±25.1
Initial phase half-life (mins)	57.5	51.4	63.0	58.3
Terminal phase half-life (hours)	61.4	64.0	56.8	53.8

Table 9. Pharmacokinetic Parameters Following Chronic Daily Oral Dosing of Normal and Adjuvant Arthritic Rats with 200 mg/kg D-Penicillamine (PSH) or 362 mg/kg Penicillamine Cysteine (PSSC). (mean ± SEM)

SECTION 4:

GENERAL DISCUSSION

4. A COMPARISON OF THE TOXICITY, PHARMACOKINETICS AND PHARMACOLOGY OF D-PENICILLAMINE IN MAN AND RATS.

The previous section has given the pharmacokinetic profiles of D-penicillamine, penicillamine disulphide and penicillamine cysteine in normal and/or adjuvant arthritic rats. Their individual pharmacodynamic effects on the development of the disease have also been assesesed. This section will compare these results with published human data and assess the suitability of rat adjuvant arthritis as a model for the screening of potential anti-rheumatic agents.

4.1 Toxicity of D-penicillamine in Man and Rats

The preliminary experiments on drug tolerance in rats as discussed in Section 3.1 have shown that, from the parameters measured, female Spague-Dawley rats aged between 6-8 weeks (120-160g) can tolerate a daily oral dose of 200 mg/kg for 18 days without any apparent adverse effects. Other animal studies have revealed strain variabilities (and hence genetic influence) in rats to the toxicity of the drug. Whilst male Lewis and Sprague-Dawley rats weighing around 150g have been reported to tolerate a daily oral dose of up to 50 mgs D-penicillamine for 8 months, male Brown Norway rats became severely ill after only 3-8 weeks treatment, characterized by weight loss dermatitis and a high

mortality rate (Donker et al, 1984).

Initial toxicological experiments also revealed that a dose of 400 mg/kg D-penicillamine induced immediate proteinuria and an increase in urinary creatinine in the young rats (Section 3.1). Doses of D-penicillamine of 400 mg/kg and above, have previously been shown to cause an immediate increase in urine volume and proteinuria in young, Sprague-Dawley rats (Khalil-Manesh and Price, 1983). This increase in urinary protein was predominantly due to albuminuria and excretion of other low molecular weight proteins. Chronic studies by the same group indicated that D-penicillamine directly affects the glomerular basement membrane of young rats, possibly by interfering with cross-link formation.

Adverse reactions to D-penicilamine therapy in man have been well documented since the drug came into clinical use in 1956. The first double-blind study to show that D-penicillamine was effective as compared to placebo (Andrews et al, 1973) used doses of 1500-1800 mg daily for 12 months. However, as well as disease improvement being evident, the trial also highlighted the relatively high incidence of complications due to D-penicillamine therapy. Since then, there have been important changes to the treatment regimen of the drug in order to improve tolerance by the patients while

reducing the side effects. The drug is now generally introduced slowly to the patient at 250 mg per day or less. This dosage can be maintained for several weeks until the natural history of the disease in the patient is discerned. The dosage may then be increased to 500 mg or up to 750 mg. A period of at least 7 days should elapse before the dose is increased as work performed recently in this department has shown that patients whose dose was increased within 7 days did not achieve steady state conditions on a particular dose (Notarianni, personal communication). Marked interindividual variability in pharmacokinetic parameters are well documented features of D-penicillamine therapy (Wiesner et al, 1981; Kukovetz, et al 1983; Bergstrom et al, 1981a; Brooks et al, 1984). However, in two studies, one measuring free drug (Brooks et al, 1984) and one measuring total drug (Muijsers et al, 1984) failed to find any correlation between D-penicillamine levels and efficacy/toxicity in patients taking 250, 500 or 750 mg of the drug. Both studies emphasised that some of the most common side effects observed with D-penicillamine therapy, proteinuria and thrombocytopenia, were not connected with high drug levels. However, it is probable that the vastly supratherapeutic doses used in the earlier trials (Andrews et al, 1973) did not lead to any further clinical improvement, but rather increased the risk of complications.

In addition to the link between dose and side effects there have a number of recent reports indicating a genetic involvement for the manifestatation of adverse reactions induced by the drug. Stastny (1978) established an association between rheumatoid arthritis and the HLA-DR4 antigen. In the same year, Panayi and coworkers (1978), suggested a genetic predisposition to toxic reactions to sodium aurothiomalate and D-penicillamine therapy. In 1980 Wooley et al, showed that the development of proteinuria in patients treated with sodium aurothiomalate or D-penicillamine is associated with the presence of HLA-B8 and DR3 antigens but found no significant association between these HLA antigens and haematological complications of treatment with either drug or both drugs. A more recent report (Perrier et al, 1985) showed that patients possessing HLA-DR3 were at a 10 times greater risk of developing skin eruptions or proteinuria after D-penicillamine therapy than DR3 negative patients. Also, studies on the genetic aspects of the polymodally distributed sulphoxidation of the mucolytic agent, S-carboxymethyl-L-cysteine (carbocysteine), in man showed that patients who were found to be deficient sulphoxidisers of the drug showed a significant increase in the manifestations of toxic reactions to D-penicillamine (Emery et al, 1984). The only enzymatic metbolism product of D-penicillamine, S-methyl penicillamine, is structurally similar to

carbocysteine. If this metabolite is responsible for toxicity it may be that patients who are poor sulphoxidisers, and hence less able to remove the drug, are more at risk from adverse reactions.

Hence it would seem that in both man and rats D-penicillamine has a great propensity to induce toxic side-effects, and the degree to which each species is at risk is related, in part, to dose and genetic background. However, whilst it seems that, in general, man only tolerates doses of D-penicillamine of less than 1000 mg (approximately 10-15 mg/kg) daily, female Sprague-Dawley rats appear to be reaction-free at daily doses of 200mg/kg.

4.2 Pharmacokinetics and Metabolism of D-Penicillamine and its Disulphides

In addition to toxicity studies, the pharmacokinetics after single and chronic oral dosing of the rats with 200 mg/kg D-penicillamine have also highlighted several species differences when compared to pharmacokinetic studies in man. In the rat, maximal drug absorption occurred at 45 minutes post-dosing. This was followed by a bi-phasic decline in serum drug concentration with an initial half-life of 27.0 mins followed by a slower decline with a half-life of 51.02 hours (single dose). After 15 days chronic oral dosing with 200 mg/kg

D-penicillamine, peak serum concentrations again occurred at 45 mins after the dose was given. However, the initial and terminal phase half-lives had increased to 51.5 minutes and 61.4 hours respectively. These values are greatly different from the pharmacokinetic parameters obtained from studies in man. Most groups report that peak serum concentrations occur at around 1-3 hours after oral dosing in man (Wiesner et al, 1981; Bergstom et al, 1981; Butler et al, 1982; Russell et al, 1979 and Kukovetz et al, 1983). A bi-phasic decay in the serum is also reported but with values of 1.5-3 hours for the rapid elimination phase and a terminal phase of between 4-6 days (Muijsers et al, 1979; van der Korst et al, 1981, Muijsers et al, 1984). Despite these differences, however, the serum levels obtained 24 hours post-dosing in the chronically treated rats are not dissimilar to those reached by patients taking 500 mg/day at steady state (Table 10, courtesy of Dr. L.J. Notarianni). It is not unreasonable to assume that patients taking 750 mg/day may easily reach levels of 19 µg/ml, as seen in the rat after 15 days chronic oral dosing with 200 mg/kg.

Due to the absence of a specific assay for the identification and quantification of the metabolites of D-penicillamine, few reports on this area of metabolism are available. From the limited studies performed to date, it would appear that in man, the major blood

Daily Dose D-Penicillamine	Plasma Concentration at Steady State (Range) µg/ml
125 mg	5.12 ± 0.24 (1.72-8.44) n=53
250 mg	8.55 ± 0.59 (5.41-14.1) n=24
375 mg	9.89 ± 0.10 (5.28-16.4) n=10
500 mg	12.94 ± 1.18 (8.60-17.2) n=7

Table 10 Total D-penicillamine Plasma Concentrations at Steady State (n = number of observations at each dose).
(mean ± S.D.)

metabolite is penicillamine-cysteine with small amounts of penicillamine disulphide and S-methyl penicillamine (Jellum and Skrede, 1976; Perrett, 1981). However, from the work performed with ^{14}C-D-penicillamine in rats, as described in Section 3.2, it would appear that in the rat, penicillamine disulphide is the predominant metabolite. Free D-penicillamine in rat serum initially accounted for 4% of the total (i.e. 11.3 µg/ml) but was undetectable 3 hours after dosing. Protein bound D-penicillamine was found to equilibrate to 55% at 1 hour after dosing, representing around 45 µg/ml D-penicillamine bound to proteins. This would suggest that the remaining 40% of the drug was present as disulphide(s). Penicillamine cysteine could not be detected, but as mentioned earlier this may be due to the lack of sensitivity of the detection system but a minimum PSSP:PSSC ratio was speculated to be greater than 14:1 (see Section 3.2). The most comprehensive study to date on the pharmamacokinetics and metabolism of D-penicillamine is by Kyogoku et al (1982) in which they measured blood and urine levels of free D-penicillamine, penicillamine cysteine and penicillamine disulphide in man, dogs and rats using a combination of HPLC and amino acid analysis. They found that in human blood the ratio of free PSH:PSSC was 1:3, and that PSSP was undetectable. However, in the rat, free PSH was predominant, with ratios of PSH:PSSC being 8:1 and PSH:PSSP being 15:1; PSSC:PSSP was around 2:1.

However, this study may be criticized in that only free D-penicillamine was measured and, as discussed previously, the method of protein precipitation with acid may release bound penicillamine which would be maintained as free thiol at the low pH. Although these results differ from the findings in this thesis, both indicate basic differences in metabolism of D-penicillamine in man and rats.

From this apparent discrepancy in metabolite formation in man and rat an investigation into how the rat handles each of the metabolites was performed. Although the experiments were limited to the measurement of total D-penicillamine in the serum and urine, it was still possible to examine the disposition of each metabolite after oral and intravenous doses. For comparitive purposes, doses of each compound were equivalent to 200 mg/kg of D-penicillamine. Although penicillamine cysteine showed considerable absorption from the gut, less than 3 % of the administered dose of penicillamine disulphide appeared in the serum. In both cases the peak serum concentrations occurred at 1 hour post-dosing, 15 minutes later than the peak observed after D-penicillamine treatment. Calculating the oral bioavailability from the AUC values after oral and intravenous administration, the bioavailabilities of D-penicillamine and penicillamine cysteine are 64.8 $\pm$ 1.6% and 48.1 $\pm$ 2.8% respectively. The value for

D-penicillamine is in accordance with other studies in rats which report figures of between 55-65% (Planas-Bohne, 1972; Ruiz-Torres and Kurten, 1974; Patzchke and Wegner, 1977). Absorption rates of D-penicillamine in man have been reported to be between 50 - 62% in the fasting situation, depending on the drug formulation (Kukovetz et al, 1983). A study by Miners (1984) on the reversible metabolism of D-penicillamine in the rat in which the presence of reduced penicillamine in the plasma after oral and iv dosing of D-penicillamine and its disulphides was measured implies almost complete absorption of penicillamine cysteine, but insignificant absorption of penicillamine disulphide. As with the Kyogoku study, (1982) free penicillamine was measured after the plasma had been acidified, which may release variable amounts of protein-bound drug leading to false reduced plasma penicillamine levels. The study also showed that D-penicillamine and its disulphides are interconvertible in the rat.This effect has also been demonstrated with captopril, which like D-penicillamine, contains a reduced thiol group and is known to form disulphide metabolites (Lan et al, 1982; Drummer and Jarrott, 1983).The enzymes involved in the reduction process are as yet unknown, but it has been suggested by Lan et al (1982) that glutathione reductase, thiol-disulphide transhydrogenases and non-specific disulphide reductases may all have a role

to play.

The pharmacokinetic data produced after oral and iv administration of D-penicillalmine and its disulphides show a bi-pasic decline in total serum D-penicillamine levels in all cases except after oral dosing with penicillamine disulphide (Section 3.3). However, the radiolabelled data reveals the existence of a third phase after intravenous D-penicillamine treatment. The half-life of this phase is extremely short, approximately 3 mins in normal rats (Section 3.2). It may be that this very rapid initial phase is the formation of disulphides, followed by an elimination phase in which free base and disulphides are excreted. The terminal, slow phase presumably represents the "deep" pool, the nature of which is as yet unknown, but may be collagen and elastin rich tissues, such as skin and bone (Patzchke and Wegner, 1977). The chronic administration data (Section 3.4) show that the terminal phase half-life of D-penicillamine in serum increases from 51.02 hours to 61.4 hours after daily oral D-penicillamine treatment and from 35.8 hours to 56.8 hours after oral dosing with penicillamine cysteine during treatment. Studies by van der Korst et al (1981) showed only slowly reversible binding of D-penicillamine to tissue compartments. It is likely, therefore, that the increase in terminal phase half-life on repeated dosing is due to the filling of

this "deep" pool from which the drug is only slowly released.

Penicillamine disulphide administered intravenously to the rat was rapidly removed from the serum and excreted in the urine, (section 3.3) approximately 70% of the given dose appearing in the urine within 48 hours of administration, almost all of this in the 0-4 hour period. In comparison, approximately 45% of the given dose of D-penicillamine or penicillamine cysteine was recovered in the 48 hours post-dosing, again the majority of which is excreted in the first 4 hours. This is consistent with the radiolabelled studies of Patzchke and Wegner (1977), who report urinary excretion rates of D-penicillamine in rats of 52% in 8 hours. It is not possible to correlate these values to urinary excretion rates in man due to the difference in route of administration of D-penicillamine. However, all studies report that an oral dose of the drug to humans results in a maximal excretion rate within 4 hours after the dose was received. Values of between 30-40% of the oral dose are reported to be recovered in the urine, incomplete absorption of the drug probably accounting for the low value (van de Stadt et al, 1979; Patzschke and Wegner, 1977).

It would appear, therefore, that penicillamine disulphide is less able to remain in the body than

D-penicillamine or penicillamine-cysteine. It is probable that in order for D-penicillamine levels to be maintained, binding to serum proteins is important, although this binding is readily reversible (van de Stadt et al, 1979). The capacity for the disulphides to undergo reduction to the free parent drug, is therefore important. Work by Theriault and Rabenstein (1985) has shown that formation of penicillamine disulphide is via a two step process:

$$PSH + CSSC \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PSSC + CSH$$

$$PSH + PSSC \overset{k_3}{\rightleftharpoons} PSSP + CSH$$

They calculated the rate constants at physiological pH to be k_1=4.66, k_2=0.024,and k_3=0.116. It would seem therefore, that <u>in vitro</u> under physiological conditions D-penicillamine will form the symmetrical penicillamine disulphide (PSSP) via penicillamine cysteine (PSSC) formation. The rate constants would indicate that the spontaneous reduction of either disulphide back to free thiol is negligible. However, the ability of the disulphides to undergo enzymic reduction is also of importance. It may be that the reduction of penicillamine disulphide is subject to steric hinderance (due to the extra methyl groups on the β carbon), moreso than reduction of penicillamine cysteine. However, some interconversion between penicillamine disulphide and free base seems to occur,

accounting for the terminal phase seen after intravenous administration. A monophasic decline in serum concentrations is seen after oral administration of the disulphide presumably because the elimination rate is faster than the rate of conversion to free D-penicillamine. In comparison, the intravenous dose kinetics seen with penicilamine cysteine are very similar to those of D-peinicillamine, differing only in a much shorter terminal phase half-life. The spontaneous formation of penicillamine disulphide in rat serum, and the relative unavailability of this compound for enzymic reduction may explain the much shorter half-lives seen in rats than in man.

4.3 The Effects of D-Penicillamine and its Mixed Disulphide (PSSC) on Rat Adjuvant Arthritis

In spite of the effectiveness of D-penicillamine in the treatment of rheumatoid arthritis, its effect in animal models is weak and variable. The failure to detect a clear-cut effect of the drug in experimental animals may be due to the difference between the major compound in the blood of rats and that of humans. It was therefore decided to examine the effect of penicillamine cysteine, generally considered to be the major metabolite in human blood, on one of the animal models of arthritis. Out of all the models described previously in Section 1.3, rat adjuvant arthritis best

suited the requirements. It is easily induced and monitored, with a high percentage of the innoculated animals developing the disease to a uniform degree. The disease time course was also reasonably short, avoiding the need for prolonged drug therapy. The effects of D-penicillamine on the development of the disease were also recorded.

Daily, oral D-penicillamine treatment (200mg/kg) from day -1 to day 18 had no effect on the various parameters used to evaluate disease severity (i.e. hind paw volumes, weight change). These observations are in accordance with the results of Liyanage and Currey (1972) who also found that the same dose of D-penicillamine failed to modify AA in rats. Similar results were also obtained by Dunn et al (1984) whereby oral pretreatment (1-3 months) of AA rats with D-penicillamine (100 mg/kg p.o) followed by daily treatment after AA induction had no effect on the progression of the disease. Indeed, in some studies, the drug has been shown to exacerbate the secondary lesions of AA (Binderup et al, 1976). Penicillamine cysteine treatment on the same schedule also failed to show any significant effects, either moderation or exacerbation, of the disease process.

Subnormal levels of serum sulphydryl groups have been observed in various connective tissue disorders

including rheumatoid arthritis (Lorber et al, 1971) and with high rheumatoid factor titres (Kosaka, 1979). A depression in serum SH levels was found in rats that developed AA, associated with the persistent chronic inflammatory phase of the disease. Maximal depression of serum SH occurred at around the time of onset of AA (day 9) although a change was noticeable as early as 1 day after AA induction. These results are consistent with the findings of other workers (Butler et al, 1969; Lorber et al, 1975). According to Lorber et al (1975) the depressed serum SH levels observed in the adjuvant rat were not due to the acute inflammatory phase or to decreased food consumption or loss of body weight. However, this depression may be caused by oxidative species, especially H_2O_2 by activated phagocytes (Hall et al, 1984) in the active inflammatory phase when gross tissue injury occurs. A similar process has been reported in rheumatoid patients (Thomas and Evans, 1975) where the depression in serum SH levels was suggested to be due to oxidation of cysteine side-chains within protein structures (predominantly albumin) with low molecular thiols such as cysteine and glutathione.

Experiments with D-penicillamine showed that although chronic treatment with the drug did not alter normal serum sulphydryl levels, it increased the serum SH levels in AA rats towards normality during the active

inflamation phase. However, it further reduced SH levels in the initial acute inflammation stage (days 1-3). Penicillamine cysteine showed a similar pattern of serial changes, further exacerbating the reduction in serum SH levels in the acute inflammatory phase. It may be that the supplementation of cysteine levels with penicillamine cysteine administration serves to further increase the oxidation of serum protein sulphydryl groups in this phase. However, in the later persistent chronic inflammation phase, it would appear, that both compounds are acting as reducing agents, restoring the free SH groups that have been blocked by other low molecular weight thiols in active AA. D-penicillamine has no effect on the mediators of acute inflammation, eg prostaglandins accounting for its lack of effect in the acute phase of the disease. This reductive ability of the compounds may be important in thiol dependent reactions of the immune system such as lymphoid cell proliferation (Broome and Jeng, 1973) and antibody responses (Click et al, 1972).

Both rheumatoid arthritis and adjuvant arthritis are characterised by high levels of serum copper (Bajpayee, 1975; Gralla and Wiseman, 1968). In this study, serum copper levels were shown to be elevated as early as day 1 after induction of AA. These levels began to fall gradually until the time of onset of the active inflammatory phase, thereafter remaining high. Urinary

copper excretion remained at a normal rate throughout the course. Although both D-penicillamine and penicillamine cysteine brought about a reduction of the serum copper levels towards normality in the acute phase, they could not totally control the increase from day 9 onwards. This serum copper reduction was reflected by the increase in urinary copper output throughout the course, a phenomenon also seen in normal rats treated with D-penicillamine. One of the characteristics of severe inflammation is the presence of "acute phase reactants" in the serum. These include elevated levels of fibrinogen, several glycoproteins and the copper-containing protein caeruloplasmin. However, there is some dispute as to whether caeruloplasmin levels are significantly increased in arthritic diseases (Pruzanski et al, 1973). The excess copper may therefore be associated with another serum component, believed primarily to be albumin, and that it is this labile albumin-bound copper that is accessible to chelating agents (Whitehouse, 1976). It is believed that in rheumatoid arthritis the penicillamine-copper chelate exerts a beneficial effect by virtue of its superoxide dismutating activity. Indeed, Sorenson (1976) has shown that copper complexes of many anti-inflammatory drugs including D-penicillamine are more active than their parent compounds in experimental inflammatory models. However, this is not reflected in this study of AA, as no

improvement of the disease was apparent.

Despite alterations in serum sulphydryl levels and protein binding in AA, the pharmacokinetics of D-penicillamine or penicillamine cysteine were not significantly different in normals than in AA rats. The initial decrease in protein binding of D-penicillamine in AA rats is probably due to the decrease in the number of available serum sulphydryl sites. This is only important, however, in the initial period after dosing, when there is a considerable amount of free D-penicillamine still present in the serum, and equilibrium is soon reached between protein bound drug and disulphide(s). Also, the results show that both D-penicillamine and penicillamine cysteine restore the serum sulphdryl levels towards normality during the AA course.

The administration of penicillamine cysteine to adjuvant arthritic rats failed to modify the disease process at all, and behaved similarly to D-penicillamine. This is contrary to a report by Nakaike et al (1983) in which they found penicillamine-cysteine to strikingly enhance the development of AA which they attributed to the elimination of suppressor cells. However, it would seem from the results described earlier, that penicillamine cysteine is merely a supply of free penicillamine and is inactive per se. In a clinical trial on D-penicillamine in 1979, Munthe et al found that the

addition of cysteine to treatment raised the level of erythrocyte glutathione and, in some cases, turned non-reponders into responders. There have also been tentatative suggestions that serum cysteine depletion is related to the incidence of D-penicillamine induced side-effects (van der Korst et al, 1981; Muijsers et al, 1984). However, this study in the AA rat has failed to reveal any beneficial effects of added dietary cysteine in the form of penicillamine cysteine.

In conclusion, it would appear that there are major differences in the metabolism of D-penicillamine in man and the rat. Penicillamiine disulphide is the major metabolite in the blood of the rat, which is less succeptible to enzymic reduction than penicillamine cysteine, the predominant metabolite in humans. The existence of the free thiol would appear to be neccessary for the maintenance of D-penicillamine in the body and the poor *in vivo* reduction of penicillamine disulphide leads to rapid excretion of the compound, hence a shorter half-life of D-penicillamine in the rat than in humans. The serum total penicillamine levels reached after 15 days chronic dosing with either D-penicillamine (200mg/kg) or penicillamine cysteine (362 mg/kg) were in the same order of rheumatoid patients taking 750 mg/kg at steady state. Therefore, the lack of response of AA to D-penicillamine treatment is not due to insufficient

serum levels of the drug, or to differences in metabolite formation. It is more likely that there are major diffences in the aetiology of rheumatoid and adjuvant arthritis, which questions the usefulness of AA as a suitable model for the screening of potential anti-rheumatic drugs.

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APPENDIX

TIME hrs.	TOTAL [D-PEN] ug/ml oral	i.v.
0.25	47.6±3.3	272.0±1.5
0.5	44.6±1.7	127.4±11.6
0.75	72.9±1.5	
1.0	54.0±2.0	80.1±13.2
1.5	39.6±6.5	39.1±3.9
2.0	33.6±3.8	26.4±4.2
3.0	24.2±0.7	24.1±1.6
4.0	23.9±1.5	19.5±4.4
5.0	20.5±0.3	18.5±4.5
6.0	16.7±4.1	12.9±2.7
7.0	15.2±1.1	11.9±0.6
8.0	15.1±0.9	14.8±0.1
10.0	18.1±0.9	11.8±1.0
24.0	13.1±1.1	10.3±0.2
32.0	15.2±1.0	9.8±1.1
48.0	9.9±0.6	11.0±2.9
56.0	9.8±0.2	6.5±0.4
72.0	6.9±0.3	7.1±0.9

Appendix 1. Total serum D-penicillamine concentration after an oral and i.v. dose of 200 mg/kg D-penicillamine. (n=3, mean±S.E.M)

TIME hrs.	TOTAL [D-PEN] ug/ml oral	i.v.
0.25	2.5±0.1	231.1±10.9
0.5	3.2±0.6	94.2±6.4
0.75	3.9±0.9	
1.0	4.7±0.7	39.4±1.9
1.5	1.4±0.1	20.9±3.4
2.0	0.4±0.1	25.3±2.4
3.0	---	7.2±1.1
4.0	---	4.6±0.5
5.0	---	5.2±0.9
6.0	---	4.7±0.7
7.0	---	4.9±0.1
8.0	---	4.6±0.6
10.0	---	3.7±0.7
24.0	---	3.7±0.8
32.0	---	2.8±0.1
48.0	---	3.5±0.2
56.0	---	3.1±0.3
72.0	---	2.2±0.1

Appendix 2. Total serum D-penicillamine concentration after an oral and i.v. dose of 202 mg/kg penicillamine disulphide (n=3, mean±S.E.M)

TIME hrs.	TOTAL [D-PEN] ug/ml oral	i.v.
0.25	14.6±0.6	226.0±16.6
0.5	25.2±5.9	156.1±1.5
0.75	28.6±4.1	
1.0	55.7±5.7	39.0±9.9
1.5	36.4±2.7	39.6±5.5
2.0	22.1±1.6	31.4±3.9
3.0	24.4±2.5	40.2±2.8
4.0	20.4±2.8	22.0±1.7
5.0	18.2±0.6	21.0±1.9
6.0	15.8±1.4	21.4±2.2
7.0	21.6±2.6	22.3±1.9
8.0	12.6±0.5	20.1±2.1
10.0	11.5±2.1	14.3±1.0
24.0	10.4±0.7	9.5±0.8
32.0	5.7±0.2	9.5±0.2
48.0	8.7±0.3	9.6±1.1
56.0	4.4±1.1	9.3±0.6
72.0	4.2±0.3	6.2±0.5

Appendix 3. Total serum D-penicillamine concentration after an oral and i.v. dose of 362 mg/kg penicillamine cysteine (n=3, mean±S.E.M)

TIME hrs.	TOTAL [D-PEN] ug/ml normal	AA
0.25	99.3±4.9	79.5±8.4
0.5	99.6±13.4	78.2±6.2
0.75	130.6±7.2	121.4±11.4
1.0	95.5±7.9	90.6±10.7
1.5	54.9±10.8	55.6±4.2
2.0	56.1±7.6	50.1±3.8
3.0	44.1±13.5	40.2±6.4
4.0	34.0±4.7	39.6±1.9
5.0	32.6±2.8	30.2±2.4
6.0	28.7±0.9	25.7±0.8
7.0	19.2±1.7	20.6±1.1
8.0	26.3±2.4	21.2±1.1
24.0	18.4±1.5	22.2±0.7
48.0	15.4±1.9	16.4±1.5
56.0	15.7±2.2	17.1±1.9
72.0	15.8±1.7	16.0±0.7

Appendix 4. Total serum D-penicillamine concentration in normal and AA rats after the last oral dose of 200 mg/kg D-penicillamine in a 15 day oral treatment course. (n=3, mean±S.E.M.)

TIME hrs.	TOTAL [D-PEN] ug/ml normal	AA
0.25	48.1±4.2	50.1±7.8
0.5	51.6±1.7	59.2±6.4
0.75	65.5±5.6	65.4±4.8
1.0	98.7±8.6	100.5±6.7
1.5	79.9±6.4	50.7±1.8
2.0	51.1±7.8	49.8±5.4
3.0	34.6±2.7	44.2±3.8
4.0	32.1±1.7	35.3±4.0
5.0	30.4±4.5	36.7±2.7
6.0	26.2±2.4	27.1±1.3
7.0	21.6±1.9	20.0±0.9
8.0	22.0±0.9	22.8±1.2
24.0	16.4±1.3	16.4±1.5
48.0	14.3±0.7	14.7±0.8
56.0	16.2±1.4	14.5±0.5
72.0	13.1±1.0	15.1±1.2

Appendix 5. Total serum D-penicillamine concentration in normal and AA rats after the last oral dose of 362 mg/kg penicillamine cysteine in a 15 day oral treatment course. (n=3, mean±S.E.M.)